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ABSTRACT BOOK

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ABSTRACT BOOK

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The European Hematology Association (EHA) aims to promote excellence in clinical practice, research and education in European hematology.
EHA was founded in June 1992 and today – with over 3200 members from 100 countries – is a consolidated representative of European hematologists.

Our aim

- To become the official European representative of hematology and hematologists – especially where research, education and regulatory issues are concerned – and to become a conduit for European harmonization;
- To promote the creation of a highly attractive market for practitioners and researchers in Europe thus fostering the mobility of hematologists in and to Europe;
- To reach out and offer a platform to countries that wish to further develop excellence in hematology;
- To promote education, training and scientific research in hematology in Europe;
- To exchange and disseminate knowledge and scientific information in the field of hematology.

Our activities

- Organizing an annual scientific and educational congress in a major European city;
- Dissemination of medical research, both basic and clinic, through the Haematologica/The Hematology Journal;
- Collaborating with other leading organizations in the field of hematology and oncology;
- Providing postgraduate education through the annual congress, tutorials and workshops;
- Supporting junior basic and clinical researchers in the development of their careers through the EHA Fellowship Program.
- Strengthening the quality and professional status of hematology throughout Europe by accrediting scientific meetings and providing CME accounts.

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15TH CONGRESS
JUNE 10 - 13, 2010
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Word of welcome

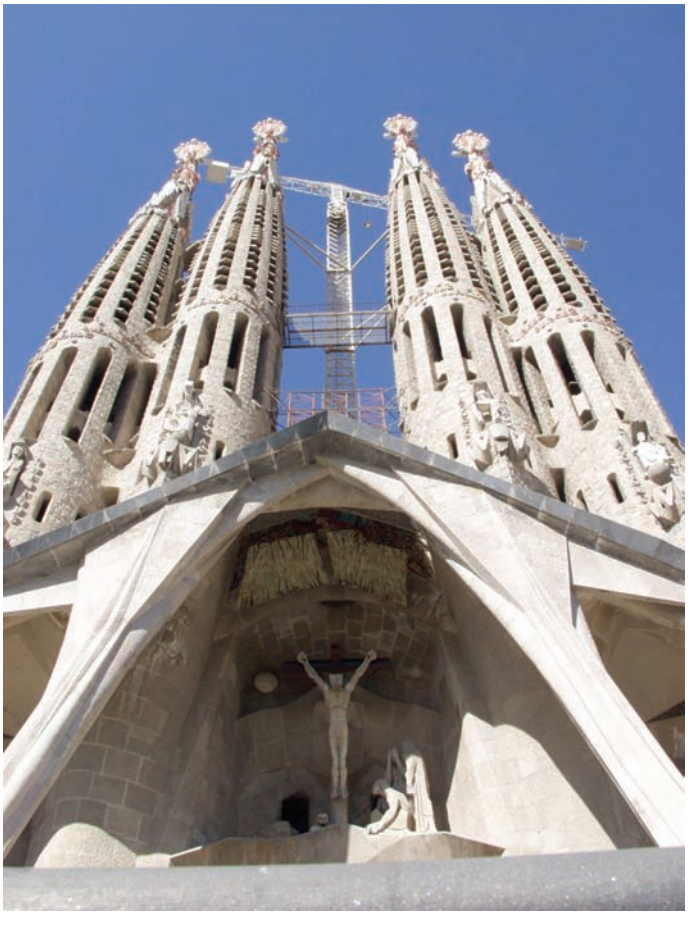
On behalf of the EHA Board and the Scientific Program Committee we would like to welcome you to Barcelona for the 15th Congress of the European Hematology Association.

This year, over 2100 abstracts have been submitted - a new record- showing the growing significance of the EHA-Congress to hematologists in Europe and abroad. The Scientific Program Committee is pleased with the steadily increasing quality of the data presented. From these abstracts, an exciting program has been composed including the Presidential Symposium (6 best abstracts), Simultaneous Oral Sessions and Poster Sessions with organized poster walks.

On behalf of the EHA Board, the committees and all people involved in this years' EHA congress, we thank you for coming to Barcelona and hope that this Abstract Book will provide you with an important reference of the recent advances in hematology research.

Christine Chomienne
Chair Scientific Program Committee

Jesús San Miguel
Congress President



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POSTER SESSION I

Acute lymphoblastic leukemia - Biology

0001

HIGH-RESOLUTION PHARMACOGENETIC PROFILES OF GENES INVOLVED IN DRUG ABSORPTION, DISTRIBUTION, METABOLISM AND ELIMINATION IN ADULT PHILADELPHIA-POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA PATIENTS

I Iacobucci,[1] A Lonetti,[1] M Sazzini,[2] S Formica,[3] A Ferrari,[1] C Papayannidis,[1] P Garagnani,[2] A Boattini,[2] A Astolfi,[3] S Paolini,[1] D Cilloni,[4] MC Abbenante,[1] V Guadaguolo,[1] A Vitale,[5] F Pane,[6] S Soverini,[1] M Vignetti,[5] R Foà,[5] M Baccarani,[1] G Martinelli[1]

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Background. Inter-individual variations in genes encoding drug metabolizing enzymes and transporters have been demonstrated to influence the response to therapy. However so far, how these genetic variations interact to produce specific drug related phenotypes in Philadelphia-positive (Ph^+) acute lymphoblastic leukemia (ALL) has not yet been investigated. *Aim.* In order to investigate potential genetic structure and related pharmacogenetic profiles, around 2000 variants in more than 200 genes involved in drug absorption, distribution, metabolism and elimination were genotyped and studied with a population genetics approach in 45 Ph^+ ALL patients. *Methods.* The Drug Metabolizing Enzymes and Transporters (DMET™, Affymetrix) platform, covering more than 90% of the most biologically relevant drug absorption, distribution, metabolism and excretion (ADME) markers was used for successfully genotyping 1931 variants in Ph^+ ALL patients treated with the tyrosine kinase inhibitor Dasatinib. A model-based clustering method for inferring population structure using genotype data was applied by means of the Structure software assuming a model in which there are K populations - each one of them being characterized by a set of allele frequencies at each locus - to which individuals are probabilistically assigned according to their genotypes. Distribution of the genetic variance observed among the identified leukemia sub-groups was investigated with a locus by locus Analysis of the Molecular Variance (AMOVA) by means of the Arlequin 3.01 package, exploiting information on genotypes allelic content and frequencies. *Results.* Three different sub-groups (G1, G2, G3), made up of 2, 12 and 31 patients respectively, were identified in the examined ALL sample, according to their different patterns of allele frequency. A statistical support for this finding was provided by AMOVA results which pointed out a substantial level of genetic differentiation among G1 and the other two sub-groups (Fst=0.099, P<0.001) and a milder but still remarkably significant differentiation between G2 and G3 (Fst=0.020, P<0.001). Fst values for each genotyped variant were also computed in order to single out those changes which were actually responsible for such statistical significances. As regards the comparison of allele frequencies among G1 and G2-G3, 56 variants, affecting the NAT1, NAT2, CYP1A2, CES2, CYP1A2, CDA, SLC22A1, CYP3A5, CYP2B6, CYP3A43, FMO2, UGT1A1, CYP3A7 and VKORC1 genes, showed very high (>0.3) and significant Fst values; whereas a total of 50 loci, located on the NAT2, VKORC1, CYP4F2, CYP2B6, UGT2B7 and CYP2D6 genes, showed moderate to high (>0.08) significant Fst values in the G2/G3 comparison. *Conclusions.* Differences of allele frequencies observed among the identified ALL sub-groups prove that an evident genetic structure is detectable in our sample by genotyping loci involved in drug metabolism.

Supported by: Fondazione GIMEMA Onlus, European LeukemiaNet, AIL, AIRC, Fondazione Del Monte di Bologna e Ravenna, FIRB 2006, Ateneo RFO grants, Project of integreted program (PIO), Programma di Ricerca Regione - Università 2007-2009.

0002

INTACT APOPTOSIS SIGNALING IN PEDIATRIC ALL IS ASSOCIATED WITH PROLONGED NOD/SCID ENGRAFTMENT, LOW EXPRESSION OF ANTI-APOPTOTIC MOLECULES AND FAVORABLE PATIENT OUTCOME

M Queudeville, F Seyfried, S Eckhoff, KM Debatin, L Meyer

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Background. Defects in cell death signaling have been attributed to be responsible for treatment failure and relapse in acute leukemia. We recently reported the importance of intact apoptosis signaling for good outcome in pediatric ALL and AML in two retrospective studies. Using a NOD/SCID mouse xenotransplant model for pediatric ALL we have also shown that rapid engraftment of primary ALL cells (short time to leukemia/TTLshort) is characteristic for early relapse of the corresponding patient. *Aims.* In this prospective study the impact of the functional integrity of apoptosis signaling in xenograft ALL samples on NOD/SCID engraftment and patient outcome was investigated. Furthermore, expression of apoptosis regulating molecules was analyzed and correlated to NOD/SCID engraftment, treatment response and apoptosis signaling. *Methods.* Primary BCP-ALL samples (N=20) obtained at diagnosis were transplanted onto NOD/SCID mice and time to leukemia (TTL) was estimated as weeks from transplant to onset of disease for each sample transplanted. Apoptosis signaling in xenograft leukemia samples was assessed by flowcytometry analyzing two key apoptogenic events, cytochrome c release and caspase-3 activation. Expression of the anti-apoptotic molecules Mcl-1, XIAP, Bcl-2 and Livin was analyzed by qRT-PCR. *Results.* Of the 20 ALL samples transplanted 6 led to rapid leukemia manifestation in the recipient animals (TTLshort) with an inferior relapse free survival of the corresponding patients in contrast to 14 patients with TTLlong phenotype (log rank P.002). Apoptosis signaling was investigated in xenograft leukemia samples by analysis of caspase-3 activation and cytochrome c release. Both events were closely correlated to each other indicating intact signaling in patients stratified into non-high risk groups, patients showing good response to treatment (remission on day 15, negative MRD on day 33), and patients without relapse. In contrast, no correlation was found in patients with poor outcome. Most importantly, intact apoptosis signaling was also found in TTLlong but not in TTLshort patients strongly indicating that mitochondrial cytochrome c release and consecutive apoptosome formation resulting in activation of downstream effector caspases such as caspase 3 is characteristic for a long engraftment phenotype and prognostic favorable ALL. The functional integrity of this apoptogenic checkpoint is subsumed by the parameter cytochrome c related activation of caspases (CRAC). Patients with positive CRAC values reflecting intact apoptosis signaling showed a significantly superior relapse free survival in contrast to patients with disturbed cytochrome c related caspase activation/negative CRAC (log rank, P<.001). Transcript expression of apoptosis regulating molecules was analyzed. A correlation of cytochrome c release and caspase-3 activation indicating proficient apoptosis signaling was exclusively observed in leukemia samples with low expression of the anti-apoptotic molecules Mcl-1, XIAP, Bcl-2 and Livin. Furthermore, patients samples with low Mcl-1 expression showed a significantly longer time to leukemia (TTL) and lower blast cells on day 8 than samples with high Mcl-1 expression. *Conclusions.* Thus, the propensity leukemia cells to undergo apoptosis (CRAC positive) leads to prolonged engraftment upon transplant in the NOD/SCID/huALL model (TTLlong), is associated with low expression of anti-apoptotic molecules, and results in favorable treatment response and superior survival of pediatric ALL patients.

0003

PHOSPHOPROTEOMIC PROFILING OF PEDIATRIC T-LINEAGE ACUTE LYMPHOBLASTIC

G Milani, B Accordi, M Giordan, S Bresolin, M Sciro, L Galla, G TeKronnie, G Basso

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Background. T-lineage Acute Lymphoblastic Leukemia (T-ALL) accounts for about 15% of paediatric ALL. Although the outcome of T-ALL has improved with current therapies, T-ALL paediatric patients remain at risk for early relapse. It is therefore very important to develop more specific and less toxic therapies through the identification of molecules, such as aberrantly activated phosphoproteins that offer the possibility to use specific kinase inhibitors. *Aim.* The aim of this research was to study the phosphorylation profile of paediatric T-ALL patients at diagnosis, through Reverse Phase Protein Arrays (RPPA), to find aberrantly activated phosphoproteins. *Methods.* We analyzed 98 paediatric patients with T-ALL using RPPA technique. This innovative approach allows to investigate post-translation modifications, such as phosphorylation, and thus to characterize the activation state of cellular protein networks. For all patients included in this study informed consent was obtained following the tenets of the Declaration of Helsinki. Twenty-seven samples were from the Padova hospital and were directly processed whereas 71 patients were from other Italian hospitals. The whole proteome of each patient was immobilized on nitrocellulose coated glass slides and each slide was stained with a different antibody, for a total of 53 antibodies. Protein expression/activation was compared between patients subgroups defined by clinical/molecular features. *Results.* We first found that the phosphorylation state of samples from Padova is comparable with that of samples from other Italian hospitals. Shipment of samples to Padova at room temperature does not influence in a systematic way protein phosphorylation, thus allowing the study by RPPA also for samples collected in other centers. Comparison between T-ALL patients with and without del(1p32) revealed that mTOR(S2448) is differentially activated between these two groups (t test with Benjamini-Hochberg multiplicity corrections P=0.003). This protein kinase that regulates cell growth and survival is less activated in 1p32 deleted patients. We then compared relapsed vs non relapsed patients. Statistical analysis revealed that PKCα(S657) is hyperactivated in the non relapsed group (t test with Benjamini-Hochberg multiplicity corrections P=0.02). Moreover, we performed a Relapse Free Survival analysis through Kaplan-Meier estimates. We searched a threshold value for PKCα(S657) that resulted in the largest difference in survival between the two groups defined by that threshold: PKCα(S657) threshold 50750.33, log rank test with Holm corrections P=0.02. Patients who show PKCα(S657) lower than 50750.33 have a higher probability to relapse: 15 of 32 (47%) patients with PKCα(S657) lower than 50750.33 relapsed, while among the 58 patients with PKCα(S657) higher than 50750.33 only (17%) relapsed. Of note, mRNA expression analysis for mTOR and PKCα did not reveal any significant difference in gene expression levels among the above studied T-ALL patients. *Conclusions.* This research identified the different activation state of proteins in subgroups of T-ALL patients with clinical relevance that may be explored for new therapies.

0004

COMBINED BCL-2 AND MTOR INHIBITION IN ACUTE LYMPHOBLASTIC LEUKEMIA CELLS

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Background. Acute lymphoblastic leukemia (ALL) is a malignant disorder of lymphoid progenitor cells. Leukemic cells resistant to induction chemotherapy lead to persistence of disease or relapse and ultimately to patients' death. We have previously observed that ABT-737 (kindly provided by Abbott Laboratories), a Bcl-2/Bcl-xL (BH3 mimetic) inhibitor, exerts potent cell growth inhibition and apoptosis induction in ALL cell lines and primary samples. It has also been reported that deregulation of mTOR signalling is frequently found in ALL and mTOR inhibition by CCI-779 is associated with anti-leukemic effects in pre-clinical models. *Aim.* Aim of the study was to investigate in ALL cell lines and primary leukemic blasts the effect on cell proliferation and apoptosis of ABT-737 and CCI-779 alone or in combination *Results.* In MOLT-4 cells, ABT-737 induced a dose and time-dependent growth inhibition (IC-50= 198 nM) followed, at higher concentrations (250-500 nM), by apoptosis induction. In contrast, CEM-S (IC-50=12.1 μM), JURKAT (IC-50=66 μM), DAUDI (IC-50=67.8 μM) and RAJI (IC-50=1.6×10^{20} μM) proved resistant. Western Blot (WB) analysis revealed that all of them share Mcl-1 overexpression, already reported as a resistant factor to ABT-737. When we explored the effects of CCI-779 on the aforementioned cell lines, only minor cytotoxic effects were found at higher concentrations (IC-50 ranging between 0.5 μM to 28.2 μM), as demonstrated in the MOLT-4 cells which showed a biphasic dose response with a flat curve (35-55% growth inhibition) at concentrations ranging between 1 nM and 5000 nM (IC50=9,87 μM). Apoptosis induction, as measured by Annexin-V positivity, was not observed until 72h at 10000 nM. Instead, cell cycle effects were noticed, as shown by a S-phase decrease, from 38.03%±3.7 (vehicle) to 27.7±7.8% at 5000nM (P=0.03). Aiming at investigating the activity of ABT-737 plus CCI-779 on the resistant phenotypes, we exposed JURKAT cells to the above combination (each of them at 1000nM). A significant (P=0.04) induction of apoptosis was found, as measured by an increase of the sub-G1 peak, to 47.7±5.9% (CCI-779+ABT-737) compared to the effects of the single agents (17.4±1.5% and 4.2±1.5% in the presence of ABT-737 and CCI-779, respectively). WB analysis revealed in the presence of CCI-779 and, particularly, its combination with ABT-737, a decrease of Mcl-1 level. Results obtained on primary cells from 8 ALL patients treated *in vitro* with of ABT-737 (ranging from 50 to 100 nM) and CCI-779 (ranging from 5000-10000 nM) alone or in combination, showed an increase of the sub-G1 peak in 5/8 and in 3/8 samples exposed to ABT-737 and to CCI-779, respectively, while synergistic effects on apoptosis induction were found in 2/8 primary samples. *Summary/Conclusions.* We observed that CCI-779 can potentiate growth inhibition and apoptosis induction of ABT-737 on ALL cells. Interestingly, the combined use of both inhibitors exerts synergistic cytotoxic effects on the JURKAT cells, mediated by the down-regulation of Mcl-1. Pre-clinical studies on an initial series of primary ALL samples, support the investigation of the active oral ABT-737 compound, ABT-263, as a novel therapeutic approach in ALL, aimed at overcoming selected resistance mechanisms.

0005

GENOME WIDE ANALYSIS REVEALS WNT11, A NONCANONICAL WNT GENE AS A TARGET OF THE ETS TRANSCRIPTION FACTOR ERG

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Background. ERG is an ETS transcription factor that is involved in leukemogenesis and mRNA overexpression is associated with poor prognosis in patients with T-cell acute lymphoblastic (T-ALL) and acute myeloid leukemia (AML). Normal *ERG* expression peaks during T-cell lineage commitment and furthermore is required for hematopoietic stem cell maintenance. Thus far, ERG transcriptional networks in leukemia are unknown. *Methods.* Herein, a genome-wide analysis of ERG target genes was conducted in a T-ALL cell line by chromatin immunoprecipitation-chip (ChIP-chip). Enriched ERG-bound DNA and total chromatin were differentially hybridized to high resolution tiled promoter chip array. *Results.* Of the significantly fluorescent annotated regions derived from duplicate ChIP-chip experiments, a total of 340 annotated genes were categorized using DAVID Gene Ontology Tools. Significant biological processes of the candidate genes with gene count out of total are listed as follows: cellular processes (232), biological regulation (118) and developmental processes (98). These data suggest a broader functional role or anomalous role for ERG in leukemia. In addition, 17 candidate promoter regions resulted in >2-fold enrichment over total chromatin by quantitative PCR. Notably, candidate genes *WNT2, WNT9A, WNT11, CCND1* and *FZD7*, of the WNT signaling pathway, were among the 17 candidate genes. Expression of *WNT11*, the most significantly enriched gene promoter by ChIP-chip, was directly reduced by 40-50% with siRNA knockdown of *ERG* (70-90% transcript level knockdown). Conversely, through a Tet-on ERG-inducible cell based system in K562 cells, *ERG* induction (>50-fold

relative to uninduced status) substantially upregulated *WNT11* mRNA expression ranging from 10 to 100-fold relative to the uninduced status from 3 independent clones. Moreover, modulation of glycogen synthase 3β (GSK-3β), a central regulator in WNT signaling, was inhibited with 6-bromoindirubin-3'-oxime (BIO) addition in the Tet-on *ERG* inducible system. *ERG*-induced cells treated with BIO (1-2 μM) elicited a growth advantage at 72 hours over uninduced BIO treated cells in a WST proliferation assay. Thus, application of GSK-3β inhibitor (BIO) confers resistance upon *ERG* induction. Lastly, ChIP of primary bone marrow samples of six newly diagnosed acute leukemia patients (one T-ALL and five AML) and one healthy bone marrow donor showed that *WNT11* promoter enrichment correlated with ERG mRNA expression. *Conclusions.* ERG transcriptional networks in leukemia are revealed in this study. Specifically, we demonstrated that ERG functionally is a positive regulator of *WNT11*. From our observation of a proliferation advantage of *ERG* induction in combination with GSK3-β inhibition, we propose a functional role for ERG as an upstream regulator in the WNT pathway.

0006

HUMAN BONE-MARROW MESENCHYMAL STEM CELLS PROTECT B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA CELLS FROM APOPTOSIS IN VITRO BY UPREGULATING NOTCH-3 AND -4 ON BOTH CELL TYPES

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Background. Notch signaling pathway plays a crucial role in promoting self-renewal in either normal hematopoietic and leukemic stem cells. In fact, deregulation of Notch signaling is involved in the pathogenesis of several forms of acute lymphoblastic leukemia (ALL), most importantly in the T-ALL with the translocation t(7;9)(q34;q34.39). However, little is known about the role of Notch signaling in B-lineage ALL. Bone marrow-derived mesenchymal stem cells (BM-MSCs) are non-hematopoietic precursors that may support hematopoietic progenitors using Jagged-1/Notch-1 signaling. *Aims.* to evaluate the role of the Notch signaling pathway in the maintenance of B-ALL cells in coculture with BM-MSCs. *Methods.* MSCs were obtained from normal BM samples collected after informed consent, and expanded up to passage 3-4, displaying a homogeneous mesenchymal immunophenotype and multipotent differentiation capability. B-ALL cells were obtained by density gradient centrifugation from BM samples of 5 newly-diagnosed patients with high blast count (mean purity: 91.2%, range: 82-97), and were cocultured with BM-MSCs at 10/1 ratio for 3, 7 and 28 days. Apoptosis of B-ALL cells was evaluated by Annexin-V/7-AAD staining, while proliferation was assessed with the Carboxyfluorescein-diacetate Succinimyl-ester (CFSE) method and flow cytometry. Expression of Jagged-1 and Notch-1, -2, -3 and -4 was assessed on both cell types with flow cytometry. *Results.* At each time point the number of surviving B-ALL cells was increased by the coculture with BM-MSCs (day 3: 56.5±1.6% vs 73.5±0.2%, P<0.001; day 7: 15.8±2.7% vs 58.0±1.4%, P<0.001; day 28: 4.6±1.5% vs 30.6±2.1%, P<0.001). This finding was due to a dramatic reduction in apoptosis rather than to the proliferation of B-ALL cells, which was negligible. At the same time, the expression of Notch-1, Notch-3, Notch-4 and Jagged-1 by B-ALL was markedly upregulated by the coculture with BM-MSCs (Notch-1: 1.7±0.6% vs 21.0±1.3%, P<0.001; Notch-3: 5.3±0.7% vs 16.3±0.7%, P<0.001; Notch-4: 1.8±0.5% vs 3.8±0.8%, P<0.001; Jagged-1: 8.4±0.7% vs 37.4±1.5%, P<0.001; all at day +3). On the other hand, Notch-3 and -4 were upregulated on BM-MSCs (Notch-3: 8.8±1.1% vs 54.8±1.1%, P<0.001 at day +3; 7.9±0.7% vs 88.5±1.7%, P<0.001 at day +7; Notch-4: 1.0±0.2% vs 5.8±0.5%, P<0.001 at day +3; 2.2±0.3% vs 48.5±1.4%, P<0.001 at day +7). Notch-2, expressed at low levels at basal conditions on both B-ALL and MSCs, became undetectable from day +3 onwards. We then blocked all the Notch signaling with the γ-secretase inhibitor XII: consequently, B-ALL cells were all dead at day +3 when cultured alone, but still 42.5±0.5% of the total population was alive by coculturing B-ALL with BM-MSCs. We next blocked single Notch receptors through specific neutralizing antibodies: 61.2±0.4%, 51.4±0.5% and 30.8±0.8% of B-ALL cells were still alive using anti-Notch-1, -3 and -4 antibodies, respectively (Figure 1). *Conclusions.* considered together, these data show how BM-MSCs contribute to the survival of B-ALL blasts by activating Notch signaling. Notch-3 and -4 appear mainly responsible for these phenomena and might become future additional targets in the treatment of B-ALL.

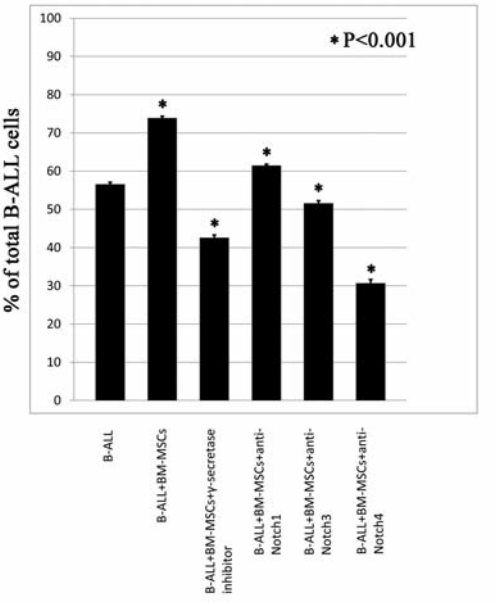

Figure 1. Viable B-ALL cells at day +3 of culture.

0007

RNAI MEDITED SILENCING REVEALS TEL/AML1 IS DISPENSABLE FOR LEUKEMIC CLONE SURVIVAL

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Backround. Translocation (12;21), the most frequent chromosomal aberration in childhood ALL results in TEL/AML1 fusion gene. TEL/AML1 hybrid protein most likely acts as an aberrant transcription factor that deregulates AML1-dependent transcription but its target genes, and thus also the exact role in leukemic cells remain unknown. *in vivo* studies showed that TEL/AML1 itself is not sufficient to cause leukemia but may induce a preleukemic state characterized by the increased numbers of multipotent or B-cell progenitors with an incomplete block of differentiation. Role of TEL/AML1 for leukemic clone establishment is being studied intensively, however, relevance of TEL/AML1 fusion gene for definitive leukemia persistance has not been approached yet. *Aims.* To address this question and to explore the potential of TEL/AML1-targeted therapy, we aimed to study the effect of RNAi-mediated TEL/AML1 silencing on leukemic cells. *Methods.* Transfection of siRNAs was used to silence TEL/AML1 and AML1 genes. HeLa cells were transfected by lipofection, REH and UOC-B6 cells (only available TEL/AML1-positive cell lines) by rectangular pulse-electroporation. TEL/AML1 level was monitored at mRNA and protein levels using qRT-PCR and western blot, respectively. Cell viability was measured using trypan blue staining followed by microscopy, apoptosis rate was monitored by staining with annexin V and propidium iodide using flow cytometry. Analysis of DNA content using staining with propidium iodide was performed to assess cell-cycle distribution. Incorporation of nucleoside analogue was measured by flow cytometry to analyse *de novo* DNA synthesis as an indicator of proliferation rate. GEP was performed in TEL/AML1-positive cells after TEL/AML1 silencing. *Results.* We designed eleven different siRNAs specifically targeting the fusion sequence to silence TEL/AML1 but prevent silencing of TEL and AML1 wild type alleles. In the first step siRNAs efficiency was measured in HeLa cells transgenic for TEL/AML1-ires2-EGFP reporter as a decrease of EGFP fluorescence by flow cytometry. In the second step five most efficient siRNAs were tested at mRNA level in TEL/AML-positive leukemic cell line. Two most efficient siRNAs were pooled and used for TEL/AML1 knock-down in REH and UOC-B6 TEL/AML1-positive cell lines. Applying two rounds of transfection within 48 hours interval we achieved 74% and 86% TEL/AML1 protein knockdown in REH and UOC-B6 cells, respectively. Seemingly counter-intuitively (based on the results from studies on other fusion oncogenes including BCR/ABL,

AML1/ETO, E2A/PBX1), TEL/AML1 silencing neither decreased cell viability, nor induced apoptosis. On the contrary, TEL/AML1 depletion was accompanied by modest but significant increase in the fraction of S-phase cells and corresponding rise in the proliferation rate. Opposite effects on cell cycle and proliferation were induced by AML1 silencing, supporting our hypothesis that TEL/AML1 may block previously demonstrated AML1 role in G1/S progression through the cell cycle. In line with the lack of effect on cell viability and discreet effect on cell-cycle distribution and proliferation we found no significant changes in global gene expression pattern upon TEL/AML1 depletion. *Conclusions.* Our data indicate, that TEL/AML1 is dispensable for leukemia persistence, at least in studied cell lines, and, as such, would not be a suitable target for gene specific therapy.

0008

CHARACTERIZATION OF THE NOVEL ABL1 FUSION TO THE PUTATIVE TUMOR SUPPRESSOR GENE SHIP1 IN ALL

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Background. BCR/ABL is the most common fusion gene in leukemia. The BCR/ABL fusion is the hallmark of CML and is frequently associated with B-lineage ALL. Up to now only 5 other mostly rare fusion partners of ABL1 have been described: ETV6, RCSD1, EML1, NUP214 and ZMIZ1. Here, we describe a novel fusion of ABL1 to SHIP1 (Inositol Polyphosphate-5 Phosphatase, INPP5D) in an 18-year-old woman with c-ALL. *Aim.* We aimed to characterize the SHIP1-ABL1 fusion at the genomic, molecular and functional level. *Methods.* Fluorescence *in situ* hybridization (FISH) analysis was performed using the BCR/ABL1 and SHIP1/ABL1 dual color dual fusion (DCDF) probes. Coimmunoprecipitations were performed by co-expressing differentially tagged versions of the SHIP1/ABL1 protein. The dimerization domain of SHIP1 was mapped using the Yeast-two hybrid (Y2H) system. Ba/F3 cells were stably transduced with a retroviral vector expressing SHIP1/ABL1 or various deletion mutants of SHIP1/ABL1. *Results.* Sequence analysis of an unexpected PCR product obtained from the diagnostic screening for a BCR-ABL1 fusion from a patient with c-ALL, revealed an in-frame fusion of the first 343 amino acids of SHIP1 to the second exon of the ABL1. The SHIP1/ABL1 fusion protein contains an SH2 domain in the SHIP1 portion (amino acids 2 to 102) and the ABL1 protein starting from exon 2. As the SHIP1 gene is located on 2q37 and is transcribed centromere to telomere like the ABL1 gene on 9q34, we predicted the SHIP1/ABL1 fusion to be on chromosome 2. Interestingly, FISH analyses using a BCR/ABL1 DCDF probe on metaphase chromosomes did not show an ABL1 signal on chromosome 2 but detected four ABL1 signals in interphase nuclei. Additional analyses with various combinations of SHIP1 and ABL1 probes revealed the presence of two SHIP1/ABL1 and one ABL1/SHIP1 fusion in the interphase nuclei from the patients. By using an anti-FLAG antibody an HA-SHIP1/ABL1 protein coimmunoprecipitated with a FLAG-SHIP1/ABL1 protein. This suggested that the SHIP1/ABL1 fusion is using a similar dimerization-dependent kinase activation mechanism like the other ABL1 fusion proteins. SHIP1/ABL1 transduced Ba/F3 cells exhibited IL3 independent growth, which was sensitive to Imatinib. Moreover, we could show that the expression of the SHIP1/ABL1 fusion protein in Ba/F3 cells leads to strong tyrosine phosphorylation of a number of proteins including STAT5a, MAPK, CRKL, and SHIP1/ABL1 itself. Using the Y2H system we were able to show that amino acids 100 to 343 of SHIP1 are necessary and sufficient for dimerization and for the induction of factor independent growth of Ba/F3 cells. *Conclusions.* Since SHIP1 functions as a negative regulator of myeloid proliferation and SHIP1 knockout mice develop a myeloproliferative syndrome-like disease, it is tempting to speculate that in addition to the activation of the ABL1 tyrosine kinase, the formation of the SHIP1/ABL1 fusion might contribute to cellular transformation by compromising the putative tumor suppressor function of SHIP1 through: 1) haploinsufficiency and 2) a dominant negative effect mediated by the interaction between SHIP1/ABL1 and SHIP1. This is the first report describing genetic lesions of SHIP1 in ALL.

0009

IL-27 ERADICATES PEDIATRIC B-ALL CELLS IN VIVO BY TARGETING TUMOR INITIATING CELLS

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Background. B-acute lymphoblastic leucemia (B-ALL) is the most common pediatric hematologic cancer which derives from aberrant expansion of early B lymphocytes in the bone marrow (BM). B-ALL is organized as a cellular hierarchy sustained by tumor initiating cells (TIC) at the apex which are the only cells with self-renewal ability, which generate both rapidly proliferating clonogenic progenitors and leukemic blasts, and maintain the bulk population of cancer cells. TIC are quiescent, slowly dividing cells and are refractory to chemotherapeutic agents that kills highly proliferating cells within the tumor. Therefore, complete eradication of this tumor may depend on the efficacy of therapies that target also TIC cells. IL-27 is a pro-inflammatory cytokine and shows anti-tumor activity in different murine solid tumors through indirect mechanisms such as immune stimulation and/or inhibition of angiogenesis. *Aims.* Objective of this study was to investigate the role of IL-27 as potential anti-tumor agent against primary B-ALL cells *in vivo* by targeting blasts and/or TIC populations. No information is available on the role of IL-27 in these cells. *Methods.* Blasts and TIC from BM of B-ALL patients after informed consent were tested for the expression of both chains of IL-27 receptor (R) (i.e. gp130 and WSX-1), by flow cytometry. The *in vivo* experiments were performed using primary B-ALL cell samples injected into SCID/NODIl2rg-/- (NOG) mice that were treated with PBS (controls) or human recomibinant (hr) IL-27. The IL-27 anti-tumor activity was evaluated in terms of tumor mass formation and spreading. Leukemic cells were searched at the primary site of tumor cell inoculation (subcutaneously and in the peripheral blood) as well as in the lymphoid organs (spleen, BM, lymph nodes) by flow cytometry and immuohistochemical analysis. In order to demonstrate unambiguously that IL-27 functions directly on B-ALL cells we cultured primary B-ALL cells in the presence or absence of hrIL-27 and tested them for apoptosis and proliferation (flow cytometry) and angiogenesis by chorioallantoic membrane (CAM) assay. *Results.* Both blast and TIC popultions expressed complete IL-27R. In the *in vivo* experiments, B-ALL cells were found in the PB, BM and spleens from controls, whereas the same cells were present in the BM only from IL-27 treated animals. The TIC population was also reduced in the BM from treated mice as compared to BM of controls. The *in vitro* experiments showed that IL-27 inhibited proliferation and angiogenesis, and induced apoptosis of B-ALL cells, demonstrating that IL-27 targets directly neoplastic B-ALL cells. *Conclusions.* Taken together, our results show for the first time that IL-27 inhibits B-ALL spreading *in vivo* and may act against both leukemic blasts and TIC. This study may open new perspectives for playing therapeutic protocols based on such double anti-tumor effect by IL-27, and aimed to obtained a complete eradication of B-ALL in pediatric patients at diagnosis and/or relapse.

0010

NOTCH3: A MORE PROMINENT PLAYER THAN NOTCH1 IN THE PATHOGENESIS OF T-ALL?

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Background. T-acute lymphoblastic leukemia (T-ALL) includes several subtypes that correlate with genetic aberrations at different stages of thymocyte differentiation. NOTCH family controls various steps of T-cell development; hence dysregulated NOTCH signaling could be involved in the development of T-ALL. NOTCH3 gene was previously shown to be expressed in all T-ALL patients, whereas its expression was reduced in remission; it is associated with the expression of HES1 and the invariant chain of pre-TCR (p-Talpha). Thus, a T-ALL signature characterizes the active or relapsing disease. NOTCH1 was discovered as a partner gene in the rare t(7;9), representing <1% of T-ALLs, yet about 50% of human T-ALLs were noted to harbor activating point mutations in NOTCH1 that lead to aberrant activation of NOTCH signaling, placing it at the center of T-ALL pathogenesis. Other studies

reported that NOTCH1 expression was not pathognomonic for T-ALL, as it was detected not only in normal peripheral blood T lymphocytes but also in non-T cell leukemias. More recently NOTCH1 activating mutations were reported as secondary events in T-ALL. *Aims.* The aim was to study the expression of the main genes involved in the NOTCH pathway, to verify the possible specific role of NOTCH1 versus NOTCH3 in the pathogenesis of T-ALL and to correlate the level of the expression of such genes to other prognostic parameters. *Methods.* Under informed consent the study was performed on 46 T-ALL patients (38 children/8 adults, 33 males/13 females); 12 cases of precursor B-ALL and 13 healthy subjects served as control groups. Gene expression was evaluated using Real Time PCR. *Results.* NOTCH1, NOTCH3, pTalpha and HES1 expression was increased in T-ALL compared to precursor B-ALL (P=0.015-0.044) and healthy subjects (P=0.0001-0.022). Genes expression was higher in children compared to adults; the difference was significant for NOTCH3 (P=0.02) and pTalpha (P=0.005). The genes level in early, intermediate and late T-ALL was examined. Due to the comparable level of gene expression in both intermediate and late T-ALL they were considered as one group in comparison with early T-ALL. Gene expression was higher in intermediate and late T-ALL group compared to early T-ALL for all the studied genes (P=0.006-0.016) except for HES1 (P=0.11). A correlation was found between NOTCH1 and NOTCH3 (r=0.508/P=0.0001) and between each of them and pTalpha (r=0.481, P=0.0001 and r=0.871,P=0.0001 respectively). HES1 moderately correlated with NOTCH1 (r=0.48, P=0.021). No association was encountered between genes expression and any of the prognostic parameters: age, TLC, mediastinal involvement, CNS involvement, hepatosplenomegaly, or lymphadenopathy except for a high expression of NOTCH1 in association with hemoglobin level <10 g/dL (P=0.049) and a negative correlation between them (r=-0.476/P=0.003). NOTCH3/NOTCH1 ratio was calculated for the different groups. Both T-ALL and precursor B-ALL showed comparable ratios to each other (P=0.312) while both showed a higher ratio in comparison to healthy subjects (P=0.0001 and 0.013 respectively) *Conclusions.* Our study confirms a pivotal role of NOTCH pathway in the pathogenesis of T-ALL together with pTalpha and HES1. The higher NOTCH3/NOTCH1 ratio suggests that NOTCH3 dysregulation may play a more central role than NOTCH1 in ALL pathogenesis.

0011

THE PROSURVIVAL CYTOKINE ANGIOPOIETIN-1 IS OVEREXPRESSED IN T(4;11)-POSITIVE ALL AND REGULATED IN A MLL/AF4-DEPENDENT MANNER

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Background. The chromosomal translocation t(4;11)(q21;q23) marks an acute lymphoblastic leukaemia (ALL) subtype prevalent in infants and associated with poor outcome. ALL cells carrying this translocation express the fusion gene MLL/AF4; in concordance with the adverse prognosis of t(4;11)-positive paediatric ALL, cell lines expressing MLL/AF4 are associated with resistance to chemotherapy-related stress and cell death. In order to address the functional role of MLL/AF4 in leukaemogenesis and leukaemic maintenance, we employ siRNA oligonucleotides targeting the fusion site of the MLL/AF4 transcript inherent of our cell line model, the t(4;11)-positive ALL cell line SEM. As previously reported by us, sustained MLL/AF4 depletion induces cell death. Gene expression profiling of the SEM cell line transfected with siMLL/AF4 revealed a subset of differentially expressed angiogenic genes, most prominently ANGIOPOIETIN-1 (ANGPT1), a proangiogenic cytokine previously reported to play a role in proliferation and survival of acute myeloid leukaemia (AML) cells, but to date not implicated in ALL. Recently, ANGPT1 signalling has been linked to haematopoietic stem cell (HSC) quiescence and bone marrow (BM) niche maintenance; pathways activated downstream of ANGPT1 include several prosurvival cascades associated with leukaemia, such as PI3K/AKT, MAPK and STAT3/5 signalling. *Methods.* ANGIOPOIETIN mRNA transcript levels were analysed by real-time RT-PCR (Q-RT-PCR), and ANGPT1 protein secretion determined using enzyme-linked immunosorbent assay (ELISA). Expression of ANGIOPOIETIN receptors and integrins was detected using RT-PCR. The MLL/AF4 status of cells was modulated using fusion transcript-specific siRNA oligonucleotides; successful knock-down was monitored by Q-RT-PCR. *Results.* Screening of a B-cell precursor ALL cell line cohort showed ANGPT1 mRNA expression to be restricted to the t(4;11) ALL subtype, where it is upregulated up to 100-fold compared to peripheral blood B cells from healthy donors. High levels of ANGPT1 were also detected in 11q23 rearranged infant ALL patient samples. Conversely, 11q23 rearranged AML cell lines show substantially lower ANGPT1 levels. Notably, in addition to its expression being restricted to t(4;11)-positive ALL, ANGPT1 levels are dependent on MLL/AF4; reduction of ANGPT1 mRNA and protein levels correlated with siRNA-mediated MLL/AF4 depletion in a time-dependent manner. Conversely, ANGIOPOIETIN-2 (ANGPT2), the biological antagonist of ANGPT1 and an independent prognostic marker in AML, showed increased RNA levels in response to MLL/AF4 knockdown in SEM cells. Expression analyses of receptors reported to mediate ANGPT1 and ANGPT2 signalling revealed presence of the integrins ITGB1 and ITGB5 in t(4;11)-positive ALL cell lines, enabling an autocrine function of secreted ANGPT1. *Conclusions.* Here we report for the first time a possible implication of ANGIOPOIETIN-1 in t(4;11) ALL, as defined by its overexpression and the regulation of mRNA and protein levels by the MLL/AF4 status of cells. ANGPT1 signalling represents an attractive potential target, both in an autocrine manner and in the context of crosstalk with the BM niche, which may promote ALL cell survival. We are currently assessing the functional role of ANGPT1 in t(4;11) ALL cells.

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0012

COMBINED INTERPHASE FLUORESCENCE IN SITU HYBRIDIZATION (CI-FISH) DELINEATES THE GENOMIC PROFILE OF T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA IN CHILDREN

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Background. In T-cell acute lymphoblastic leukemias (T-ALL) diverse molecular lesions deregulate genes involved in cell cycle control, proliferation, differentiation, survival and apoptosis. Our combined interphase fluorescence *in situ* hybridization (CI-FISH) assay reliably detected cryptic aberrations and elucidated preferential and forbidden associations of multiple genetic events concurring in adult T-ALL pathogenesis.[1] It also provided great advantages of major savings in time, costs and biological material as it combined over eight molecular assays in one slide. *Aims.* To extend our CI-FISH assay to include 30 loci/genes for a pilot study in children with T-ALL. *Methods.* We analysed 33 cytogenetic pellets of children with T-ALL who were enrolled and uniformly treated under the AIEOP LLA 2000 protocol. Immunophenotyping indicated early pre-T in 16 cases, thymic in 10, and mature in 7. Karyotyping was informative in 10 (30%), showing abnormalities in 4 (12%), but failed in the others. RT-PCR detected *MLL-ENL* and *CALM-AF10* fusion in 1 case each. *Results.* CI-FISH was abnormal in 30/33 patients, detecting ≥2 genetic changes in 20. Mono- or bi- allelic del(9)(p21)/CDKN2AB deletion was the most frequent abnormality (17 patients; 51%). In 5/17 cases del(9)(p21)/*CDKN2AB* included JAK2/9p24 and PAX5/9p13 and in 4/17 cases it included PAX5. Monoallelic del(6)(q16)/GRIK2 was found in 5 cases (15%). *TCRB*-rearrangements were detected in 5 patients (15%): inv(7)(p15q34)/*TCRB-HOXA* (2 cases), t(7;11)(q34;p15)/*TCRB-LMO1* (2 cases), t(7;9)(q34;q32)/*TCRB-TAL2* (1 case). *TCRA/D* underwent rearrangement in 3 cases: t(11;14)(p13;q11)/*TCRAD-LMO2* (2) and t(10;14)(q24;q11)/*TCRAD-HOX11* (1). Other expected changes were: del(1)(p32)/*SIL-TAL1* (8 cases), *TLX3*-translocation (5), *CALM-AF10* (3), MYB duplication (2), *MLL*-translocation, dup(9)(q34)/*ABL1-NOTCH1-NUP214*, and *PTEN*/10q24, *WT1*/11p13, and *ETV6*/12p13 deletion (in 1 case each). We detected loss of LEF1/4q25, in 2 cases, and of IKAROS/7p11, 1 case. Genomic imbalances which have still not been characterised molecularly were observed in individual cases: 1p32/SIL-TAL1 and 5q35/*TLX3* losses, 1p32/*SIL-TAL1* and 17q11/*NF1* gains. *Summary/conclusions.* Although conventional cytogenetics and RT-PCR were uninformative in the majority of T-ALL cases our new CI-FISH assay identified molecular lesions in 91% of cases and multiple changes in 60%. It provided a picture of a myriad of genomic lesions in T-ALL, the roles of which as driver or passenger hit still needs investigation. Remarkably, 73% of our cases could be classified according to well-established gene expression profiling signatures, i.e. *SIL-TAL1*; *CALM-AF10*, *MLL*, and *HOXA*; *TLX3*; *LMO1* and *LMO2*. Two broad groups of predominant associations emerged: the del(9)(p21)/*CDKN2AB* was preferentially associated with *HOX11L2*, *LMO1*, or *CALM-AF10* while the del(6)(q16)/

GRIK2 appeared preferentially associated with *TAL2* or LMO2. Approximately 18% of children had del(9)(p21)/CDKN2AB alone or associated with loss of *GRIK2*/6q16 or gain of *NF1*/17q11. In conclusion, as present findings indicate our CI-FISH assay is a valid tool in the genetic characterization of pediatric T-ALL, we propose its use in the diagnostic work-up. Its application in large prospective studies will serve to evaluate the clinical impact of a genome-based classification in this specific ALL subtype. *Aknowledgments.* PRIN 20078C9NRT_003.

Reference

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0013

PROMOTER REGION HYPERMETHYLATION CONTRIBUTES TO C-MET DOWNREGULATION IN PEDIATRIC MLL-REARRANGED BCP-ALL

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Background and Aims. c-Met is the tyrosine-kinase receptor for HGF. We previously observed that c-Met is expressed in normal B cells and in TEL/AML1-rearranged patients,[1] while its expression is strongly reduced in MLL-rearranged BCP-ALL patients. c-Met forms a protein complex with Fas in TEL/AML1-rearranged leukemias, and the activation of the c-Met pathway was demonstrated to result in increased Fas-mediated apoptosis. This implies that c-Met has an important role in cellular homeostasis and enhanced pharmacological response of TEL/AML1 BCP-ALL cells, and c-Met downregulation in MLL-rearranged leukemias could contribute to chemotherapy resistance observed in these patients. We decided to investigate by which mechanisms c-Met transcription is regulated in BCP-ALL with particular attention to MLL-rearranged cells, first of all focusing on the methylation status of the c-Met promoter. *Methods.* For all patients included in this study the informed consent was obtained following the tenets of the Declaration of Helsinki. We analyzed c-Met expression by SybrGreen RQ-PCR in 22 TEL/AML1-, 14 MLL-rearranged patients and the two human cell lines REH (BCP-ALL with TEL/AML1) and RS4;11 (BCP-ALL MLL-rearranged). REH and RS4;11 cells were treated with 5-aza-dc to test c-Met expression induction by demethylation treatment. To determine the methylation status of the c-Met promoter region in patients (15 TEL/AML1, 13 MLL) and cell lines we then performed bisulfite modification, Methylation Specific PCR (MS-PCR) and sequencing. Quantification of methylation status was performed by measuring relative peak heights in MS-PCR sequencing.[2] *Results.* TEL/AML1-rearranged patients express c-Met more than MLL-rearranged ones (Wilcoxon test, P=0.0001). Demethylating treatment of 72h induced c-Met expression by 150 times in RS4;11 cells, while only 15 times in REH cells. MS-PCR demonstrated that RS4;11 cells are completely methylated, while REH cells are only partly methylated. All MLL-rearranged patients show both methylated and unmethylated bands, while 2/15 TEL/AML1-rearranged patients are completely unmethylated. To better define the difference in the methylation status between MLL- and TEL/AML1-rearranged patients, the degree of methylation of the 6 CpG islands included in the MS-PCR methylated amplicones were quantified by MS-PCR sequencing. We measured the relative peak heights of C (corresponding to methylated C) and T (corresponding to unmethylated C) at the CpG sites. The methylation degree of TEL/AML1-rearranged patients was relatively even (17-50%) at the 6 CpG sites, whereas the methylation level of MLL-rearranged specimens ranged from 45-89%. *Conclusions.* As expected from our preliminary results, c-Met expression was found to be downregulated in MLL-rearranged human BCP-ALL. Responsible of this downregulation appears to be, at least in part, a higher methylation degree of its promoter region. As to our knowledge this is the first study reporting c-Met methylation levels in human cancers. This research will give new insights on the molecular mechanisms at the base of chemotherapy resistance observed in MLL-rearranged patients.

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THE GERMLINE STEM CELL SELF-RENEWAL GENE PIWIL2 IS EXPRESSED IN BOTH MALIGNANT AND NON-MALIGNANT LYMPHOID CELLS

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Background. The *PIWI-Like* genes (*PIWIL1-4*) are human homologues of the highly conserved family of PIWI-PAZ Domain genes. The protein products of this family perform a central role in endogenous RNA interference pathways. However, PIWI-Like proteins and their non-coding RNA partners, known as PIWI interacting RNA's, function via novel and poorly understood mechanisms, distinct from classical RNA interference. In health, *PIWIL2* is believed to be expressed only in germline stem cells where it is essential for self renewal. More recently, *PIWIL2* expression has been identified in a number of human malignancies raising the possibility that it is involved in the self-renewal programme of cancer stem cells. This gene has been designated as cancer-testis antigen 80. *Aims*. This project aimed to investigate the role of *PIWIL2* in the self-renewal programme of cancer stem cells in childhood acute lymphoblastic leukaemia. Unexpected expression of *PIWIL2* in normal peripheral leukocytes extended the scope of the project to include a wider range of normal leukocyte populations. *Methods*. Following sequencing of the *PIWIL2* amplicon to ensure specificity, quantitative RT-PCR was used to assess expression of the *PIWI-Like* genes. Expression in the t(4;11)(q21;23) positive cell line SEM was modified by transfection of cells with siRNAs specific for the *PIWIL2* transcript. Healthy donor leukocyte populations were purified by immunomagnetic cell separation to provide $CD3^+$ T lymphocytes, $CD19^+$ B lymphocytes, $CD14^+$ monocytes, $CD15^+$ granulocytes. Purity was assessed by flow cytometry, complemented by morphological analysis. *Results*. Expression of *PIWIL2* was demonstrated in a range of acute leukaemic cells lines and primary leukaemic blasts. Expression in the SEM cell line was knocked-down following transfection with *PIWIL2* specific siRNA. Initial studies suggest a substantial proportion of *PIWIL2* transcript may be retained within the nucleus, limiting overall knock-down. Peripheral lymphoid cells from five healthy donors (three male) showed consistent expression of *PIWIL2* and *PIWIL4* (known universal expression), whilst *PIWIL1* and *PIWIL3* were not expressed. Monocytes maintain expression of *PIWIL2*, although to a lesser degree than lymphocytes. Terminally differentiated granulocytes showed no expression of *PIWIL2*. *Conclusions.* Expression of the germline stem cell self-renewal gene *PIWIL2* in cell line and primary acute lymphoblastic leukaemia specimens suggests that this gene may have a role in the self-renewal of leukaemic stem cells. Originally, we hypothesised that the specificity of this gene for germline stem cells and malignant diseases would make it an attractive target for novel therapy development. However, the unexpected finding in the present study - that mature lymphoid cells, a population known to possess substantial potential for clonal expansion and self-renewal also expresses *PIWIL2* - raises doubt over the suitability of *PIWIL2* as a therapy target. Furthermore, the potential role of *PIWIL2* in other mature tissue stem cells should be considered. This work will now be extended to assess the expression of PIWI-Like genes in $CD34^+$ haematopoietic stem cells where we hypothesise *PIWIL2* will play a role in self-renewal.

0016

SQSTM1: A NEW PARTNER FOR NUP214 IN ADULT T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. NUP214, a FXFG nucleoporin located on the cytoplasmic side of the nuclear pore complex (NPC), plays a critical role in cell cycle progression and import/export trafficking. In T-cell acute lymphoblastic leukemia (T-ALL) *NUP214* fusions appear to predict a poor prognosis. So far two *NUP214* fusions were identified in T-ALL: when cryptic del(9q) is present SET fuses with *NUP214*, while episomal amplification leads *NUP214* to rearrange with ABL1. Supervised gene expression profiling (GEP) analysis in adult T-ALL identified *SET-NUP214* positive cases with high expression of HOXA cluster genes, MEIS1 and NUP214, and low expression of *SET, FNBP1*. *C9orf78* and *USP20* genes.[1] Among 69 cases of T-ALL, an additional case emerged at GEP analysis with over-expression of *HOXA, MEIS1, NUP214*, and also of *SET*. This 20-year old man with a chemo-resistant pre-T ALL showed 46,XY, del(6p) karyotype at diagnosis. *Aims.* To investigate last case as an hypothetical new rearrangement involving NUP214 in T-ALL. *Methods.* We set up FISH assays with RP1-112N13 for the 9q sub-telomere, RP11-143H20, flanking the *3'NUP214*, RP11-544A12, spanning the gene, and two overlapping fosmids, G248P89801E11 (exons 25-31) and G248P8659A12 (exons 31-36). Additional FISH experiments were done with 5q35-qter probes: RP11-117L6, RP11-286C20, RP11-549A4, RP11-319K2, RP11-718N2, RP1-240G13. NESTED RT-PCR was performed using primers SQSTM1_ex3_528F (5'-TGCCCAGACTACGACTTGTG-3') / NUP214_ex36_6543R (5'-AGTAATCATGCGCCTTGTGAGTT-3'), for the first amplification round, and SQSTM1_ex4/5_763F (5'-AATCAGCTTCTGGTCCATCG-3')/NUP214_ex33/34_6337R (5'-CAAAGCTGAACCCTCCTGTG -3') for the second. PCR products were cloned into pGEM-T easy vector system and sequenced. *Results.* FISH probe RP11-143H20 gave two signals on normal chromosomes 9 while RP11-544A12 and RP1-112N13 gave 3 signals on chromosomes 9 and on der(5). The *NUP214* breakpoint fell between G248P89801E11, which was retained on chromosome 9, and G248P8659A12, translocated to der(5). The 5q35 breakpoint was telomeric to RP11-718N2, a ~1.7Mb region containing 15 candidate genes, with an appropriate centromere-telomere orientation. GEP indicated down-regulation of genes telomeric to *SQSTM1*, which was thus selected as first candidate gene. RT-PCR detected an amplification product of 852bp. Molecular cloning and sequencing identified the in-frame fusion between nucleotide 849 (exon 5) of *SQSTM1* and nucleotide 6014 (exon 33) of *NUP214*. Nested-PCR and molecular screening of 60 T-ALL adults did not identify any additional case. *Conclusions. SQSTM1* point mutation and over-expression were described in congenital Paget's Bone Disease and solid tumours, respectively. No translocations involving *SQSTM1* have been reported, yet. In this adult with T-ALL *NUP214/9q34* rearranged with *SQSTM1* as a result of a cryptic unbalanced translocation with an apparently normal chromosome 5, i.e, ish der(5)t(5;9)(q35;q34). As in other NUP214 leukemic fusions, the FG repeats were maintained, suggesting similarities with leukemic recombinations of other nucleoporins such as NUP98. However, together with the NUP214 FG repeats, the SQSTM1 N-terminal structural and regulatory motifs, such as the Zinc-finger domain may contribute to the leukemogenic process. To elucidate the incidence and the clinical impact of *NUP214* rearrangements in T-ALL we strongly recommend *NUP214* FISH screening in large prospective studies. DDG and PG shared co-authorship. *Aknowledgements*. PRIN 20078C9NRT_003

Reference

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0017

INSULIN RECEPTOR SUBSTRATE 1 (IRS1) AND 2 (IRS2) SIGNALING IN HUMAN ACUTE LEUKEMIAS; A POSSIBLE ROLE OF IRS SIGNALING IN THE LEUKEMIA PHENOTYPE

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Background. Insulin-like growth factor 1/Insulin Receptor Substrates (IGF-I/IRS) signaling pathway plays an important role in the development of various cancers such as breast, colon and prostate. In ALL Philadelphia-positive, IRS1 expression was noted to be overexpressed and correlated negatively with survival. IRS proteins, including IRS1 and IRS2 play an important role in mTOR/P70S6K and MAPK pathways, through their interaction with effectors such as PI3K, SHP2 and Grb2. PI3K and Grb2 activation leads to higher ERK1/2 and mTOR phosphorylation, respectively, culminating in a proliferation increase. Despite both IRS-1 and IRS-2 being two important adaptor molecules essential for intracellular signaling of insulin and IGF-I, the distinct biological pattern of IRS-2 versus IRS-1 and their roles in acute leukemia remains an interesting issue to be clarified. *Aims.* To investigate IRS1 and IRS2 expression in leukemia cell lines and AML and ALL patient bone marrow samples. We also evaluated the effects of IRS1 knockdown, by lentiviral vector, in MAPK and mTOR signaling pathways in K562 cell line. *Methods.* Myeloid (HL60, P39, KG1, K562, NB4) and lymphoid cell lines (Jurkat, MOLT-4, Raji and Daudi) were used. Primary leukemia cells were obtained from bone marrow of 22 AML and 10 ALL patients. Normal hematopoietic cells were obtained from bone marrow of 07 healthy donors. The National Ethical Committee Board approved the study. Real-time RT-PCR analysis from total bone marrow cells and western blot from mononuclear cells were performed to determine differential expression of IRS1 and IRS2. Specific shRNA-expressing lentiviral vector to IRS1 gene was used in K562 cell line. Western blot and immunoblotting with specific antibodies was used to evaluate ERK and P70S6K expression and phosphorylation. *Results.* IRS1 gene and protein expression were observed in all leukemia cell lines studied, whereas IRS2 gene and protein were preferentially expressed in myeloid cell lines. Decreased IRS1 and IRS2 gene and protein expression was observed in primary AML cells compared to normal hematopoietic cells. The relative gene expression of IRS1 was 0.40 [1.41-0.10] for AML versus 0.009 [7.14-0.008] for normal cells (P=0.03); of IRS2 was 4.53 [9.37-1.00] for AML versus 0.98 [4.34-0.10] for normal cells (P=0.007). IRS1 and IRS2 phosphorylation was not detectable in AML samples. In ALL samples, IRS1 expression was not significantly different from normal hematopoietic cells (0.60 [11.64-0.008] versus 0.40 [1.41-0.10], P>0.05). IRS2 expression was decreased in primary ALL compared to normal cells (0.11 [0.53-0.01] versus 4.53 [9.37-1.00], P<0.0001). Interestingly, IRS1 phosphorylation was slightly increased in ALL samples compared to normal cells. IRS1 silencing (65% of inhibition) in K562 cells resulted in decreased ERK and P70S6K phosphorylation. *Conclusions.* IRS1 and IRS2 are differentially expressed in AML and ALL cells. The up-regulation and phosphorylation of IRS1 in ALL cells suggests an important role of IRS1 in the leukemia phenotype. In K562 cells (Philadelphia-positive cells) lacking IRS1, the decreased phosphorylation of ERK and P70S6K indicates a down-regulation of MAPK and mTOR pathways. Further studies would be necessary to better elucidate the role of IRS1 and IRS2 in leukemogenesis. This work was supported by FAPESP and CNPq.

0018

EVI1 OVEREXPRESSION ROLE IN ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. The ecotropic viral integration site 1 (EVI1), located in chromosome 3q26, has been recognized in the last years as one of the most aggresive oncogenes associated with neoplastic diseases of myeloid cells. The inappropiate expression of EVI1 without rearrangements of chromosome 3q26 has been implicated in the development or progression of Acute Myeloblastic Leukemia (AML). Correlation between overexpression of EVI1 and high risk AML is a frecuent discussion issue in the literature. In our knowledge, EVI1 overexpression has not been associated with lymphoid pathology and its prognostic value in Acute Lymphoblastic Leukemia (ALL) has not been evaluated yet. *Aims.* To analyze the incidence of EVI1 expression in ALL and its value as a prognostic factor. *Methods.* The study was performed restrospectively on a total of 48 patients with ALL. Expression of EVI1 gen ($EVI1^+$) was examined in bone marrow samples and/or peripheral blood at diagnosis by QRT-PCR, adapting the technique developed by M. Russell et al (Blood 1994) to the LightCycler 480 system, using as a fluorescent tracer SybrGreen I. Survival curves were plotted following the Kaplan Meier method and differences between the curves were analyzed with the Log Rank test. *Results.* Out of 48 patients, 12 overexpressed EVI1 (26,1%). We also analyzed other cytogenetic lesions that are frequently seen in association with $EVI1^+$. Results showed that within 12 cases $EVI1^+$, 3 patients were Ph^+ (25%), deletion of chromosome 7 was found in only 1 case (8.3%), translocations involving 11q23 in 2 cases (16.6%) and a normal caryotype in 3 cases (25%). 3q26 chromosmal abnormalities weren't found. In contingent-valuation study (Fisher test) associations between EVI1 overexpression and Philadelphia cromosome were not observed. In the $EVI1^+$ group the complete remission was achieved up to a 67% of the patients compared to the 86% achieved in the EVI1- group (p (Mantel-Haenszel)= 0.098). Survival curves didn't show any significant differences in overall survival (OS) and disease free survival (DFS) when compared the $EVI1^+$ and EVI1- population, though we observed an initial higher mortality in the EVI^+ group. Neither significant differences in OS and DFS were observed when compared EVI^+/Ph^+ and EVI^+/Ph^- population. *Conclusions.* 1) Our study shows for the first time EVI1 overexpression in a high percentage of ALL. 2) No relationship was found between the expression of EVI1 with normal caryotype or other cytogenetc alterations previously related to EVI1. 3) There is a trend toward a lower achievement of CR in the EVI1+ group compared with the EVI1- group, without statistical relevance. This may be due to the low number of samples in this study. It seems that EVI1 overexpression doesn't have an important role in the OS, DFS or relapse in ALL patients.

0019

IN PEDIATRIC LYMPHOBLASTIC LEUKEMIA OF B CELL ORIGIN A SMALL POPULATION OF PRIMITIVE BLAST CELLS IS NON-CYCLING, CONSISTENT WITH LEUKEMIA STEM CELLS

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Background and *Aims.* In pediatric precursor-B cell acute lymphoblastic leukemia (PBC ALL), the majority of blast cells express the B cell antigen CD19. However, a minor fraction of blast cells lack CD19 expression corresponding to a more primitive cell population. Investigations on cell proliferation in pediatric ALL are usually based on the entire leukemic cell population reflecting the proliferative behavior of the predominant $CD19^+$ blast cells. In light of the ongoing debate on the immunophenotype of leukemia stem cells in pediatric PBC ALL, we analyzed the proliferative characteristics of ALL cells at different stages of immunophenotypic maturation. *Methods.* Isolated mononuclear bone marrow cells of 16 children with untreated PBC ALL were stained with monoclonal antibodies against CD19 and CD3 and then separated by fluorescence-activated cell sorting (FACScan Becton-Dickinson). The sorted cells were incubated *in vitro* with bromo-deoxy-uridine (BrdU) and, after preparation of cytocentrifuge smears, stained with an anti-BrdU antibody to enable detection of cells in the S phase of the cell cycle (BrdU-LI). In addition, individual smears of sorted CD19-CD3- cells were stained with monoclonal antibodies against myeloperoxidase (MPO), glycophorin A (GLA), CD34 or CD10. Because the data were non-normally distributed median and interquartile ranges (IQR) were calculated. *Results.* Bone marrow cells were sorted into $CD19^-CD3^+$, $CD19^+CD3^-$ and $CD19^-CD3^-$ populations. Whereas the $CD19^+CD3^-$ cell compartment represented a pure blast cell population, the $CD19^-CD3^-$ compartment was composed of both leukemic blast cells and residual normal hematopoietic cells. Therefore, smears were stained for MPO and GLA to exclude normal hematopoietic cells. By staining of sorted $CD19^-CD3^-$ cells for CD34 or CD10 the compartment of these blast cells was further subdivided. The relative frequencies and the BrdU-LI of these subpopulations are shown in the Table. *Conclusions.* In untreated pediatric PBC ALL the continuous expansion of the leukemic cell population is exclusively based on the proliferative activity of $CD19^+$ blast cells. The very low proliferative activity of primitive $CD19^-$ blast cells is consistent with self-renewal but not with expansion. Due to their immature immunophenotype and their low proliferative activity, $CD19^-$ blast cells may represent leukemia-initiating cells. Whereas actively proliferating $CD19^+$ cells may be killed by current treatment approaches, the primitive non-cycling population may survive and subsequently contribute to disease relapse.

Table.

Immunophenotype	Frequency in % median (IQR)	BrdU-LI in % median (IQR)
All leukemic cells	100	7.1 (5.3-8.1)
$CD19^+$ leukemic cells	97.5 (96.2-98.3)	7.2 (5.7-8.8)
$CD19^-$ leukemic cells	2.4 (1.8-3.5)	0.2 (0.1-0.5)
$CD19^-CD34^+$ leukemic cells	1.4 (0.6-2.8)	0.2 (0.1-0.4)
$CD19^-CD34^-$ leukemic cells	0.8 (0.5-1.6)	<0.1 (0-0.1)
$CD19^-$ leukemic cells	2.4 (1.8-3.5)	0.2 (0.1-0.7)
$CD19^-CD10^+$ leukemic cells	1.2 (0.6-2.1)	0.3 (0-0.7)
$CD19^-CD10^-$ leukemic cells	1.3 (0.4-2.0)	<0.1 (0-0.2)

0020

$CD34^+CD38^-$ LEUKEMIC STEM CELL PROFILE CORRELATED WELL WITH THE CYTOGENETIC AND MOLECULAIRE FEATURES IN ACUTE MYELOIDE AND LYMPHOIDE LEUKEMIA AT DIAGNOSIS

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Background. Acute leukemia is thought to arise from a rare putative 'leukemic stem cell'(LSC) that is capable of self-renewal and formation of leukemic blasts. CD38 is expressed in acute lymphoblastic leukemia (ALL) and in acute myeloide leukemia (AML) and its prognostic significance is still unknown. *Aims.* We investigated CD34 and CD38 expression in 93 AML and 73 ALL adults and paediatric patients, and we asked if the frequency of LSC at diagnosis is correlated with specific cytogenetics or molecular events and with the CR and survival. *Methods.* Diagnosis of leukaemia was based on FAB and WHO classifications. Blasts

were identified by CD45dim/SSC characteristics. The "LSC profile" was defined as % of $CD34^+CD38^-$ in $CD34^+$ gated cells and based on rMFI of CD38 from all $CD34^+$ and $CD45^{dim}$/SCC gated cells. *Results.* CD38 was lower in AML-M3 compared to other FAB subtypes. Interestingely, we observed that higher frequency of $CD34^+CD38^-$ was more associated with secondary AML or AML-MRC (myelodysplasia-related changes), but was similar among the primary AML. We show that reduced relative mean fluorescence of CD38 on $CD34^+$ cells correlate well with the cytogenetics unfavourable and intermediate risk groups in the adults AML (P 0.082 or P 0.014 for variance test). The Ph^+ ALL patients showed significant lower CD38 expression than in nonPh^+ patients (P 0.0032). Overall survival favored AML and ALL patients with higher CD38 levels. *Conclusions.* Our results showed that frequency of LSC profile at diagnosis could be predictive for cytogenetics and moleculaire features in acute leukaemia. Screening of $CD34^+CD38$p-value cells is a simple flow cytometry method that might be considered to design a multicolor panel setting in parallel with MRD evaluation. The goal in leukaemia therapy is to eradicate LSC and targeting them after reaching CR should be required. The possibility to detect and characterize LSC should help to reach this goal.

0021

DIVERSE ASPARAGINE SYNTHETASE EXPRESSION IN LYMPHOID BLASTS IS NOT RELEVANT TO THE SENSITIVITY TO L-ASPARAGINASE

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Background. Acute lymphoblastic leukaemia (ALL), the most common haematological malignancy in childhood, is treated by combined chemotherapy, which includes the enzyme L-Asparaginase (L-Asp). The cytotoxic effect of L-Asp consists in its ability to deplete extracellular asparagine and glutamine. The sensitivity of primary ALL cells to this depletion is traditionally explained by decreased activity of glutamine-dependent enzyme asparagine synthetase (ASNS). Despite the fact that increased ASNS level was indeed shown to be connected with L-Asp resistance, the exact relationship between ASNS expression and L-Asp sensitivity has not been elucidated so far. Glutamate dehydrogenase (GDH) is an enzyme necessary for glutamine synthesis. Microarray analysis showed significantly lower expression of GDH gene in primary TEL/AML1$^+$ blasts in comparison with TEL/AML1[-], which might lead to deficiency of glutamine in these cells and consequently higher sensitivity to L-Asp. *Aims.* The aim of this study was to pursue the association of ASNS gene expression, protein level and sensitivity to L-Asp in ALL cell lines and primary paediatric ALL samples. We also focused on the role of GDH in the L-Asp mechanism of action. *Methods.* ALL patient samples and 4 leukemic cell lines were used in this study: Nalm6 (TEL/PDGFRB$^+$); RS4;11 (MLL/AF4$^+$); REH (TEL/AML1$^+$) and UOCB6 (TEL/AML1$^+$). The gene expression was determined by qRT-PCR, ASNS protein content was detected by Western Blot. The L-Asp sensitivity was evaluated by MTS assay. Gene knock-down was performed by electroporation of specific siRNA. *Results.* We showed that ASNS protein levels reflected ASNS mRNA levels and these. correlated negatively with L-Asp sensitivity. UOCB6 as the most resistant cell line (IC_{50}=0.04U/mL) had the highest expression of ASNS (normalized ASNS, nASNS=4.946), followed by NALM6 (IC50=0.01U/mL; nASNS=1.8), REH (IC50=0.6×10^{-4}; nASNS=1.176) and RS4;11 (IC50=0.3×10^{-4}; nASNS=0.024). Detection of protein content in primary ALL blasts was not possible due to significantly lower (2log) ASNS gene expression compared to cell lines. We were interested in how the sensitivity to L-Asp is affected by ASNS gene silencing. Gradient knock-down was realized in 2 ALL cell lines: REH with intermediate basal expression and RS4;11 with very low basal expression. A gradual silencing of ASNS gene in REH cell line led to gradual increase of L-Asp sensitivity till the level of 50%. Further knock-down did not influence the L-Asp sensitivity. The reduction of ASNS did not potentiate L-Asp cytotoxicity in RS4;11 cell line with primary lowest ASNS expression. We found lower GDH gene expression in primary TEL/AML1$^+$ compared to TEL/AML1$^-$ leukaemias (P=0.019). Silencing of GDH gene in TEL/AML1$^+$ REH cell line increased L-Asp sensitivity. *Conclusions.* Our data demonstrate that in cells with very low ASNS expression, as shown in primary ALL blasts of various subtypes, the difference in ASNS levels is not relevant to the sensitivity to L-Asp. We identified GDH as a new player in the response to cytotoxic effect of L-Asp. Furthermore, we suggest a relationship between ASNS and GDH based on our observations of increased GDH expression in cells with silenced ASNS gene.
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0022

PROMOTER METHYLATION ANALYSIS IN CHILDHOOD T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA AND HEALTHY T CELL SUBSETS

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Background. T-cell acute lymphoblastic leukemia (T-ALL) is a heterogeneous subtype of acute lymphoblastic leukemia with cure rate of approximately 70%. Significant effort is currently being aimed at better understanding of T-ALL biology. This could contribute to better subclassification of patients into risk groups and, in result, it could lead to improved therapy adjustment. DNA methylation seems to be a possible disease delineating marker. Different authors note that the DNA methylation status changes in various tumours, compared to healthy tissues. Methylation patterns are known to be specific for tumour types and hence, potentially, could be used as a tumour marker. *Aim.* We investigated promoter methylation status of 16 genes known to be involved in T-ALL pathology (NFkB, CDKN1C, SYK, CDH1, sFRP1, NES1, CDKN1B, p73, FBXW7, ASPP1, ADAMTS5, DIABLO, WIF1, RB1, CDKN2A, LATS1). *Patients and Methods.* The study group consisted of 54 consecutive children with T-ALL treated at 11 centers of Polish Pediatric Leukemia and Lymphoma Study Group (PPLLSG). We also analyzed peripheral blood T cells from 11 healthy children at similar age as controls. Additionally, we tested thymocytes at different developmental stages pooled from 5 healthy donors: DN1, DN2, DN3, ISP, $DP3^-$, $DP3^+$, SP $CD4^+$, SP $CD8^+$, TCRαβ and TCRγδ. Patient, control and thymic DNA was first modified by sodium bisulfite and then, promoter regions of each gene of interest were analyzed by methylation specific PCR (MS-PCR). *Results.* The CpG methylation pattern in the study group of T-ALL patients was altered when compared to the control group of healthy children, showing hypermethylated promoter regions in 7 out of 16 analyzed genes (43%) (sFRP1, CDH1, NES1, WIF1, CDKN1C, DIABLO and SYK). However, when T-ALL group was compared to thymic subsets, the CpG methylation pattern was altered in promoter regions of only 3 of 12 (25%) genes analyzed until now. When T-ALL group was compared to thymic subsets, hypermethylation was observed for ADAMTS5 gene (in 57% of patients), CDH1 (50% patients), CDKN1C (26% patients). No change in methylation pattern was observed for sFRP1, DIABLO, CDKN1B, ASPP1, p73, FBXW7, LATS1 and RB1. CDKN2A seems to be hypomethylated in T-ALL compared to healthy T cell subsets. Additionally, methylation status seems to be changing depending on the developmental stage of thymocyte. *Conclusions.* The results of our study indicate that DNA hypermethylation occurs in T-ALL, which largely seems to reflect the differentiation stage arrest, at which maturation of T cell entering tumor transformation was stopped. However, we postulate that altered methylation patterns of several genes (ADAMTS5, CDH1, CDKN1C) might be related to T-ALL pathogenesis and might possibly be useful for T-ALL subclassification and, in the future, for better therapy adjustment.

0023

QUANTITATIVE ASSESSMENT OF WT1 EXPRESSION FOR MONITORING MINIMAL RESIDUAL DISEASE IN ACUTE LEUKEMIA PATIENTS

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Wilms' tumor 1 (WT1) gene encodes a transcriptional factor involved in normal cellular development and is abnormally expressed in many types of haematological malignancies. It is over-expressed in most cases of acute myeloid leukemia (AML). *Aims.* Our study aims to estimate the usefulness of WT1 expression quantitative assessment for monitoring MRD and for relapse prediction in acute myeloid leukemia and in acute lymphoblastic leukemia (ALL). *Methods.* Real time quantitative RT-PCR was made according to the Europe Against Cancer Protocol. WT1 transcript level was determined in BM samples of adult patients with AML (except acute promyelocytic leukemia) and ALL. We examined the samples of 65 patients with AML and 33 patients with ALL at diagnosis. We also analyzed 106 specimens of AML patients and 100 specimens of ALL patients during follow-up. The obtained WT1 values were normalized with respect to the βGUS transcripts. *Results.* The median value of WT1 levels at diagnosis was 649 (range 1,6-8462) in AML patients and 1188 (range 1,5-14879) in ALL patients (no statistical difference in Student's t-test). The median value of WT1 levels of patients in complete remission was 4,3 (range 1,1-9,7) for AML and 7,5 (range 1,0-36,5) for ALL (P=0,05) . At diagnosis 91% of AML patients and 61% of ALL patients showed the expression of WT1 one log higher than the median values in complete remission. There were 2 patients (10%) with BCR-ABL (one p190 and one p210) within the ALL subgroup with high WT1 expression at diagnosis whereas within the ALL patients with low WT1 expression at diagnosis were 7 ones (54%) with BCR-ABL (6 patients with p190 and 1 with p210) (10% versus 54%, P=0,02 in chi-sguare test with Yate's correction). We had 6 patients with T-ALL, all of them showed the over-expression of WT1 at diagnosis. In the patients bearing a fusion gene transcript we performed a simultaneous analysis of WT1 and fusion-gene transcript expression: in 15 patients with AML (7 AML1-ETO, 1 CBFβ-MYH11, 2 BCR-ABL, 1 DEK-CAN, 4 MLL duplications) and in 15 patients with ALL (9 BCR-ABL, 3 MLL-AF4, 1 MLL-ENL, 2 E2A-PBX1). It was found the parallelism between the dynamics of WT1 level and fusion-gene transcript level during follow-up. The haematological relapse was correlated with the increase of WT1 expression: 8 AML patients had a median of 1545 (range 140-2578) and for 7 ALL patients a median value was 263 (range 8,0-2665) in relapse (no statistical difference in Mann-Whitney U test). We observed that the increase of WT1 level was occurred earlier than the relapse was stated for AML patients and also for ALL patients with high WT1 expression at diagnosis. Except one AML patient with BCR-ABL (p190): the increase of fusion gene expression was occurred in haematological remission without the increase of WT1 level. Conclusion. Quantitative assessment of WT1 expression may be useful for detection of MRD and can predict imminent relapse during follow-up in patients without additional molecular markers in AML and in ALL with high WT1 expression at diagnosis.

Acute myeloid leukemia - Biology 1

0024

IDENTIFICATION OF CRITICAL TUMOR SUPPRESSOR GENES (TSGS) AND NEW PROGNOSTIC FACTORS IN HUMAN MYELOID DISORDERS USING RETROVIRAL INSERTION MUTAGENESIS

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Background. Although a major improvement in diagnostic tools for AML and MDS has been established in the past few years, the exact (epi)genetic defects underlying these diseases are still unknown in many patients. Genome-wide methods (e.g. gene expression profiling, arrayCGH and methylation profiling) are currently being used to better understand the etiology of these diseases. Although these techniques generate invaluable information, it remains difficult to discriminate "drivers"contributing to the pathogenesis of these disorders from "passengers, which do not. Other approaches, as retroviral integration mutagenesis in mice have been proven powerful to identify "driver"genes which play an essential role in human myeloid malignancies. *Aims.* We aimed to identify new candidate TSGs in human myeloid malignancies. This was achieved by developing a new approach to retroviral integration mutagenesis to enrich for TSGs in combination with extensive comparative analysis in human AML. *Methods*. Genomic DNA of 6 murine tumors, induced by Graffi 1.4 murine leukemia virus, was isolated and digested. Methylated viral integration sites (mVIS) were determined using a combination of methylated DNA immunoprecipitation (MeDIP), inverse PCR and promoter array hybridisation. Genes flanking mVIS were identified using CEAS and their human orthologues were determined in HomoloGene. Gene expression and arrayCGH data of a *de novo* AML cohort were used for further comparative studies and DAVID was used for functional annotation analysis. *Results.* We identified 615 known (e.g. CDKN2A and CDKN2B) and new candidate TSGs. Interestingly, 149 candidates were down regulated in 454 *de novo* AML patients compared to 11 normal CD34 positive counterparts. These were enriched for genes involved in cell cycle regulation, specifically with functions in mitotic spindle formation (MAD2L2, PRC1) and genomic stability (H2AFX, SMC1A, DDX11 and DDX12). Secondly, we identified 271 genes down regulated within one or multiple AML subgroups (defined by the WHO). Strikingly, these were enriched for genes involved in DNA damage and repair, indicating that down regulation of different critical genes all may lead to a suboptimal function of DNA damage and repair pathways within different AML subgroups. Additionally, we identified 56 candidate TSGs located at small regions affected by deletion causing LOH in one or multiple AML cases, 30 of which (53,6%) were located on chromosome 7. Loss of chromosome 7 is usually seen in MDS progression towards AML and in AML with a poor prognostic outcome. Although minimal affected regions are identified, it remains unclear which are the critical genes on chromosome 7 that contribute to leukemogenesis. The 30 identified genes are potential candidates playing a role in this process. Interestingly, the relevance of the study was even further illustrated by the identification of 4 TSGs candidates with prognostic value in AML. *Conclusion.* By using a new approach to retroviral integration mutagenesis in mice and extensive comparative studies in human AML, we identified many new potential TSGs. Not only the enrichment of these genes for essential molecular pathways, as DNA damage and repair, but also the identification of new potential prognostic factors demonstrate the high potential of this screen.

0025

CHARACTERISATION OF AML1-ETO9A LEUKAEMIOGENESIS USING AN INDUCIBLE MURINE MODEL

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Background. Acute Myeloid Leukaemia (AML) is dependent on a small subfraction of cells, termed Leukaemia Initiating Cells (LICs) (or "Leukaemia Stem Cells", LSCs). These cells display the ability to self-renew and initiate, maintain or regenerate the disease. Recent studies have demonstrated that within the bone marrow (BM) not only a cell

subset with an immature HSC-like phenotype, but also committed myeloid progenitors can acquire LIC function to sustain AML. One of the common genetic abnormalities associated with around 12% of *de novo* AML involves the t(8;21)(q22;q22) chromosomal translocation, which generates the AML1-ETO (AE) DNA-binding fusion protein. Studies in mouse models have demonstrated that additional secondary mutagenic events are required for AE leukaemogenesis. However, the truncated AML1-ETOtr (AEtr) and AML1-ETO9a (AE9a) C-terminal AE isoforms have been shown to promote AML in mouse retroviral transduction-transplantation models. Thus, AE9a seems to play a key role in the multistep model of AML pathogenesis. *Aims.* To better define the role of AE9a as an initiating mutation we aim to develop an AE9a inducible murine model of AML. This system will allow us to investigate at different disease stages the effect of AE9a on LICs and on disease progression. *Methods.* We have generated a tet-on mouse line using Embryonic Stem (ES) cells with a hemagglutinin (HA)-tagged-AE9a-IRES-GFP inducible cassette. AE9a mice were also backcrossed with p53-null mice. To restrict the expression of AE9a to the haematopoietic system bone marrow (BM) cells from the generated mouse lines were used in transplantation assays to reconstitute sublethally irradiated recipients following doxycycline treatment. Alterations of the haematopoietic system were investigated by flow cytometry, haematological and histological analysis and molecular assays. *Results.* Our data suggest that AE9a-expressing murine cells display enhanced replating ability in methocult cultures *ex vivo*. The replated cells confer an immature immunophenotype but are not able to dramatically expand. *in vivo* AE9a induction demonstrates the presence of a GFPlowLin+/-cKit-/low cell population, as well as the emergence of a GFPhiLin-Sca1-cKit$^+$ immature, premalignant subpopulation, which progressively increases over time. This GFPhi cell subset emerges initially in the BM following infiltration of the peripheral blood (PB) and spleen and disappears after dox withdrawal. Preliminary experiments suggest that this cell population cannot be transplanted in secondary recipients. Interestingly, AE9a enforced expression in a p53-null background can lead to a more rapid accumulation of the GFPhi subpopulation following disease development with a latency of 21-48 days. *Summary/Conclusions.* Altogether, our data demonstrate that although AE9a impairs normal haematopoiesis, it can not induce AML without additional mutations. Thus, the proposed AE9a inducible model could be a useful tool for the dissection of the multistep pathogenesis of leukaemia and for the better understanding of the LIC origin.

0026

LOSS OF RKIP IN ACUTE MYELOID LEUKEMIA WITH A MONOCYTIC PHENOTYPE

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Background. RAF kinase inhibitor protein (RKIP) has been described as a negative regulator of the RAS-mitogen activated protein kinase (MAPK) signaling cascade. Several solid neoplasms show decreased or absent expression of RKIP and its role as a metastasis suppressor has been firmly established in animal models and studies of human tumors. *Aims.* As constitutive activation of the RAS-MAPK pathway occurs frequently in acute myeloid leukemia (AML), we investigated the involvement of RKIP in the pathogenesis of this disorder. *Methods.* RKIP protein expression was analyzed by Western blot and subsequently correlated to AML subgroups as represented by the French-American-British (FAB) classification. Results were confirmed using a microarray data set of 285 AML patients (GEO accession GSE1159). Stable transfection of RKIP was performed in THP-1 and 32D cells and soft agar colony formation assays were performed. Proliferation was measured by BrdU-incorporation and apoptosis by Caspase-3 and PARP cleavage, respectively. Differentiation assays were performed in HL-60 AML cells by incubation with 1,25 dihydroxyvitamin D3 (1,25 D3) following RKIP overexpression or knockdown. *Results.* Twenty-one of 96 (22%) AML patients' samples and five of 19 (26%) AML cell lines, but none of ten purified CD34$^+$ hematopoietic stem and progenitor cell specimens exhibited complete or partial loss of RKIP. To test the biological consequences of RKIP loss in AML, we examined the effect of introducing an RKIP transgene in the monocytic AML cell line THP-1, which is characterized by decreased RKIP expression. Re-expression of RKIP induced a decrease in cell numbers, a finding that was confirmed in murine 32D hematopoietic cells. Reduced cell numbers were due to a decrease in proliferation, rather than increased apoptosis. Importantly, the oncogenic potential of THP-1 cells - as assessed by colony formation in soft agar - was significantly diminished following RKIP reconstitution. When we correlated RKIP expression with morphological criteria of leukemic cells in 96 AML patients, RKIP loss was significantly correlated to subgroups with a monocytic phenotype (FAB types M4 and M5; $P = 2.13\times10^{-7}$). This finding could be corroborated in a previously described transcriptomic data set of 285 AML patients ($P = 1.6\times10^{-8}$). To test for a causative role of RKIP silencing in the development of a monocytic phenotype, we performed cell differentiation assays using the immature AML cell line HL-60. Its monocytic differentiation, induced by treatment with 1,25 D3, was paralleled by significant RKIP downregulation. Concomitant knockdown of RKIP with siRNA further increased differentiation, whereas RKIP overexpression resulted in inhibition of maturation indicating that RKIP is cooperating in the development of a monocytic AML phenotype. *Summary/Conclusions.* Loss of RKIP is a frequent molecular event in AML and highly associated with a monocytic phenotype. In contrast to solid tumors, RKIP acts as a tumor suppressor in hematopoietic cells and is causally contributing to the development of a monocytic phenotype of AML blasts.

0027

DIFFERENTIAL RESPONSES TO CXCR4 ANTAGONISM IDENTIFY TWO BIOLOGICAL DISTINCT SUBTYPES OF ACUTE MYELOID LEUKEMIA IN TRANSPLANTED NOD/SCID MICE

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Background. Stromal cell-derived 1 (SDF-1)/CXCR4 interactions participate in the development of leukemic blasts in acute myeloid leukaemia (AML) influencing their trafficking, survival and differentiation. *Aims. and Methods.* We used the xenotransplantation model of nonobese diabetes/severe combined immunodeficiency rcnull (NOG) mice reconstituted with human primary cells from AML patients and 2 small molecule competitive antagonists of CXCR4, AMD3100 and TN140 to better define the role of CXCR4/SDF-1 on the leukemic blast burden within the bone marrow and in extramedullary sites. *Results.* We first investigated the correlation between CXCR4 expression or function on leukemic blasts and their ability to engraft the bone marrow of NOG mice in 34 patients. Using flow cytometric analyses, we observed that CXCR4 membrane expression was highly variable between patients. In most cases, the cells expressed relatively low levels of CXCR4. This expression did not correlate with engraftment. In addition, SDF-1 responsiveness evaluated by transwell migration only marginally correlated with engraftment. However, in these initial analyses, we observed that the differences between engrafters and nonengrafters were significant if the cut-point migration is set to 20%. Patients with higher chemotactic response to SDF-1 had a significantly increased NOG repopulating ability. In order to evaluate the importance of CXCR4/SDF-1 axis in AML development, NOG mice reconstituted with cells from 6 different patients were treated with optimal concentration of either AMD3100 or TN140 for 1 week. We observed that CXCR4 inhibition by TN140 (used as a single therapy) had profound inhibitory effects on the proliferation and the development of extramedullary dissemination of the disease in this xenotransplantation for 3 patients. These patients had initially high CXCR4 level on blasts. *Conclusions.* These findings demonstrate that CXCR4/SDF-1 axis contribute to organ infiltration in AML with extramedullary leukemia. In addition, CXCR4 blocking agents effectively antagonize SDF-1 promoting leukemic development in selected patients characterized by high CXCR4 expression.

0028

HIGH BRE EXPRESSION IS ASSOCIATED WITH T(9;11)(P21;Q23) AND A FAVORABLE OUTCOME IN PEDIATRIC ACUTE MYELOID LEUKEMIA

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Background. One important cytogenetic subgroup of pediatric acute myeloid leukemia (AML) is characterized by translocations of chromosome 11q23, which accounts for 15-20% of all cases with an evaluable chromosome analysis. In most of these cases, the mixed lineage leukemia (MLL) gene is involved. More than 50 fusion translocation partners of the MLL gene have been identified and outcome differs by translocation partner, suggesting differences in the biological *Background. Aim.* In the present study, we used supervised analysis of gene expression profiles to identify and analyze differentially expressed genes between the different MLL-rearranged AML cases by translocation partners that may underline the observed differences related to outcome. *Methods.* We used microarrays to generate gene expression profiles of 245 pediatric AML cases, including 53 MLL-rearranged cases. To find gene expression signatures characteristic for the different groups, an empirical Bayes linear regression model was used. *Results.* Using these profiles, we were able to identify a specific gene expression signature for t(9;11)(p22;q23), the most common 11q23 rearrangement in AML, and identified BRE (brain and reproductive organ-expressed) as one of the genes to be discriminative for t(9;11)(p22;q23) (P<0.001). The mean average VSN normalized expression for BRE in the t(9;11)(p22;q23) subgroup was 3.7-fold higher compared with that in other MLL-rearranged cases (P<0.001). Validation by RQ-PCR confirmed this higher expression in t(9;11)(p22;q23) cases (P<0.001). In addition, we confirmed that overexpression of BRE was predominantly found in t(9;11)(p22;q23) in an independent gene expression profile cohort (Ross et al, Blood 2002). Remarkably, patients with high BRE expression showed a significant better outcome for 3 year disease free survival (pDFS) (80±13 vs. 30±10%, P=0.02) within the MLL-rearranged AML. Moreover, multivariate analysis, including, age, WBC and favorable karyotype, showed that next to favorable karyotype, BRE overexpression is an independent favorable prognostic factor within pediatric AML for DFS (HR=0.2, P=0.03). However, *in vitro* studies did not identify differences in cell proliferation, apoptosis or drug sensitivity for overexpression of BRE. *Summary/Conclusions.* Recently, overexpression of BRE has been described in hepatocellular and oesophageal carcinomas. However, to the best of our knowledge, BRE had never been associated with hematological malignancies before. Our study shows that overexpression of BRE is predominantly found in MLL-rearranged AML with a t(9;11)(p22;q23). Moreover, high BRE expression is an independent favorable prognostic factor due to a reduced relapse rate in remission. So far, we could not elucidate the exact underlying mechanism, but *in vitro* experiments did not provide evidence for differences in cell proliferation, drug sensitivity or apoptosis. Further research is warranted to explore this and to identify the link between MLL-AF9 and the transcription of BRE. Our data show that within pediatric MLL-rearranged AML specific factors, i.e. translocation partner and overexpression of specific genes, can refine risk stratifications and the development of novel therapeutic targets.

0029

DIFFERENTIAL ROLE OF MYELOID TRANSCRIPTION FACTORS, C/EBPα AND PU.1 IN LEUKEMOGENESIS BY MLL-FUSION ONCOGENES

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Background. MLL translocations found in acute leukemia possess unique clinical characteristics. They have over 50 different fusion partners and show poor prognosis. These MLL fusion proteins lost H3K4 methyltransferase activity of wild-type MLL, but gained the ability to induce aberrant expression of HoxA cluster genes. Moreover, these proteins are able to transform hematopoietic stem/progenitor cells into leukemic stem cells (LSCs). Previous studies have shown that C/EBPα and PU.1, well-known myeloid specific transcription factors, were common molecular targets of myeloid malignancies. We and others have recently shown that C/EBPα and PU.1 are negative regulators of hematopoietic stem cells, suggesting that these transcription factors may play a role in the generation of LSCs. Because we have little knowledge on the role of C/EBPα and PU.1 in MLL-leukemia, we asked whether these key myeloid transcription factors are involved in the leukemogenesis by MLL-fusion proteins. *Aims.* We aimed to clarify the role of C/EBPα and PU.1 in the leukemogenesis induced by MLL-fusion oncogenes. Notably, we focused on the role of these transcription factors during the stages of leukemia initiation and progression. *Methods.* 5-FU (150 mg/kg) treated bone marrow (BM) cells from C57BL/6J mice were harvested, pre-stimulated with recombinant mouse (rm) SCF, rmIL-6, rhFL, rhTPO (50 ng/mL each), then transduced with pMYs-IG-MLL-ENL or pMXs-IG-MLL-Septin6. These cells were serially replated in methylcellulose, then transferred to rmIL-3 (10 ng/mL) containing liquid culture (immortalized cells), or were transplanted into lethally irradiated recipients (primary leukemic cells). MLL-ENL (or MLL-Septin6) immortalized cells or MLL-ENL primary leukemic cells were transduced with pMXs-IRed-C/EBPα-ER or pMXs-IRed-PU.1-ER. After sorting of GFP+DsRed+ cells, these cells were serially replated in methylcellulose with or without 4-hydroxytamoxifen (4-HT) (1 μM), or were treated with or without 4-HT (1 μM) for 5 days, then transplanted into sublethally irradiated secondary recipients. BM cells from PU.1 +/- mice, or E14.5 fetal liver (FL) cells from PU.1 knockout or +/- mice were harvested, pre-stimulated with rmSCF, rmIL-6, rhFL, rhTPO, then transduced with pMYs-IG-MLL-ENL. These cells were also transplanted into lethally irradiated recipients. *Results.* Overexpression of PU.1, but not C/EBPα, suppressed the serial replating capacity of the cells immortalized by MLL-ENL and MLL-Septin6. Moreover, activation of PU.1 suppressed the propagation of MLL-ENL leukemic cells in the secondary transplanted animals. In contrast, activation of C/EBPα did not eradicate leukemic cells in the same settings. PU.1 deficiency perturbed leukemia development by MLL-ENL. Haploinsufficiency of PU.1 delayed leukemia development by MLL-ENL and prolonged survival of the recipients. *Summary/Conclusions.* These results indicate that the dosage of PU.1 activity has profound impact on the self-renewal of LSCs and *in vivo* leukemia formation induced by MLL-fusion oncoproteins. Therefore, PU.1 may serve as a potential therapeutic target for MLL-leukemia.

0030

TET2 MUTATIONS OCCUR IN OVER 20% OF ADULTS WITH DE NOVO CYTOGENETICALLY NORMAL ACUTE MYELOID LEUKEMIA, AND THEIR PREVALENCE INCREASES WITH AGE: A CANCER AND LEUKEMIA GROUP B STUDY

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Background. Mutations in the *tet oncogene family member 2* (TET2) gene occur in various myeloid malignancies, including myelodysplastic syndromes, myeloproliferative neoplasms, and secondary and *de novo* acute myeloid leukemia (AML). The frequency and age distribution of *TET2* mutations and their association with clinical characteristics and other molecular markers have not been defined in *de novo* cytogenetically normal (CN-) AML. *Aims.* To determine the incidence and spectrum of *TET2* mutations and their association with patient characteristics and outcomes in a relatively large cohort of *de novo* CN-AML patients. *Methods.* Pretreatment marrow or blood samples from 339 *de novo* CN-AML patients (age range, 18-81 years [y]) enrolled on cytarabine/daunorubicin-based CALGB protocols [CALGB 8525, 8923, 9420, 9621, 9720, 19808, 10201] were studied for mutations in TET2 and a panel of oth-

er genetic alterations (*NPM1, WT1, CEBPA,* and *IDH1/IDH2* mutations, *FLT3* internal tandem duplications and tyrosine kinase domain mutations, MLL partial tandem duplications). All coding exons of *TET2* (GeneBank accession NM_001127208) were PCR-amplified from genomic DNA; mutations were identified by direct sequencing. Known single nucleotide polymorphisms, synonymous sequence changes, and intronic variations were not considered as mutations. *Results.* Overall, 101 *TET2* mutations were found in 75/339 patients (22%): 46 patients had single heterozygous mutations, 24 patients had two, and one patient had three mutations. In the four remaining patients, mutations appeared to be homozygous or hemizygous, suggesting loss of heterozygosity at the *TET2* locus. Of the 101 mutations, 38 were frameshift mutations, 29 missense mutations, 26 nonsense mutations, two in-frame insertions/deletions, and 6 affected splice sites. We did not identify "hot spots"for nonsense and frameshift mutations, which occurred throughout all coding exons. However, the majority of missense mutations and in-frame changes (22/31 mutations) clustered in the two evolutionarily conserved regions of the gene. *TET2* mutations were significantly more common in CN-AML patients aged ≥60y (41/150; 27%) than in patients <60y (34/189; 18%) (P=.048). The proportion of TET2 mutated patients gradually increased with age, from 10% in adults <30y to 31% in patients aged ≥70y. Moreover, the proportion of patients with double *TET2* mutations was also higher among patients ≥60y (18/41; 44%) than among younger patients (7/34; 21%) (P=.049). We observed no correlation between the presence/absence of any *TET2* mutation and other pretreatment characteristics (leukocyte and platelet counts, hemoglobin level, blast percentage, FAB category, or extramedullary involvement). Both *IDH1* and *IDH2* mutations were less common in *TET2* mutant than in *TET2* wild-type patients, although the difference only reached statistical significance in patients ≥60y, where *IDH1/IDH2* and *TET2* mutations were mutually exclusive (IDH1, P=.006; IDH2, P<.001). No significant association with other molecular markers and no impact of *TET2* mutations on clinical outcome (complete remission rate, disease-free, or overall survival) were observed in either age group. *Summary*. *TET2* mutations are relatively frequent in *de novo* CN-AML patients, and their prevalence increases with age. The presence of *TET2* mutations is inversely associated with *IDH1/IDH2* mutations but not with other molecular markers or clinical characteristics, and seemingly does not affect outcome.

0031

BONE MARROW FAILURE IN ACUTE MYELOID LEUKEMIA IS INDUCED BY A BLOCK IN DIFFERENTIATION OF NORMAL HEMATOPOIETIC STEM CELLS

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Background. Bone marrow failure (reduced production of neutrophils, platelets and erythrocytes) is an important cause of morbidity and mortality in leukemia. The mechanism of bone marrow failure in AML (Acute myeloid leukemia) is believed to be different to that seen in lymphoid malignancies such as chronic lymphocytic leukemia (CLL). In AML bone marrow failure occurs early in the disease course. By contrast, in CLL the blood count may be relatively preserved even where the majority of cells in the marrow are leukemic. *Aim.* Understand the mechanism of bone marrow failure in AML. *Methods.* Two approaches were used to study bone marrow failure: 1) Six primary AML samples were transplanted into immunodeficient mice to see the effect of the AML on normal residual hematopoietic cells. Immunophenotyping was used to assess numbers of normal mouse differentiated cells (mouse $CD45^+$ Lineage marker$^+$), normal progenitors (mouse $CD45^+$ Lineage$^-$ $CD117^+$) and normal hematopoietic stem cells (HSCs) (mouse $CD45^+$ Lineage$^-$ $CD117^+$ $CD150^+$ $CD48^-$) within the mouse bone marrow. Mice were sacrificed at different time points to allow us to follow the evolution of marrow failure. Colony forming assays and transplant assays were used to complement the immunophenotyping. 2) We have recently shown that the $CD34^+$ fraction of certain types of AML is normal and contains normal colony forming units (thought to equate to normal progenitors) and normal SCID-repopulating cells (thought to equate to normal HSCs)(Taussig et al Blood 2010). We used these types of AML to study the numbers of normal HSCs ($CD34^+$ $CD38^-$) and progenitor cells ($CD34^+$ $CD38^+$) in the bone marrow at diagnosis. *Results.* Within the immunodeficient mice two phases were observed as the AML developed. In the first phase, the numbers of normal differentiated cells and normal progenitors were significantly reduced while the number of HSCs was not significantly different to controls (Figure 1). Functional assessments using colony forming assays and secondary transplants confirmed the phenotyping data. Annexin-V (a marker of apopotosis) staining on normal hematopoietic cells from mice transplanted with AML was not significantly different to controls. In the second phase the numbers of HSCs, progenitors and differentiated cells were all reduced. Within humans the number of normal progenitors was reduced by approximately 10 fold in the bone marrow of AML patients (P<0.05) while the number of normal HSCs was not significantly different to control (Controls n=28, AML patients n=12). These effects were similar to that seen in the first phase in the mice. The data are consistent with a block in differentiation of normal HSCs. *Conclusions.* AML induces bone marrow failure by blocking differentiation of normal residual HSCs in a mouse model of AML and in humans. By further understanding this mechanism it may be possible to reverse this process and ameliorate the effects of bone marrow failure.

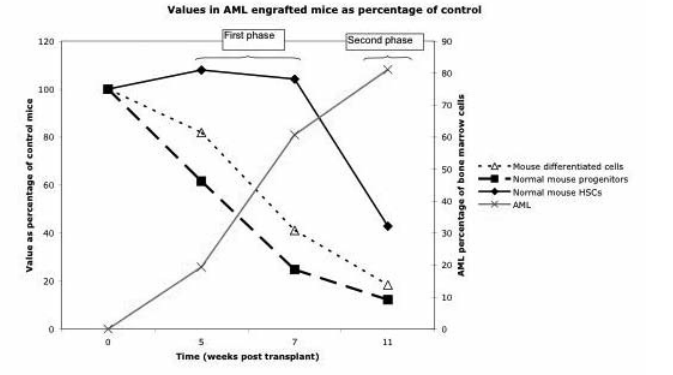

Figure 1.

0032

INFLUENCE ON OUTCOME OF THE NPM1/FLT3-ITD MOLECULAR STATUS FOR AML PATIENTS IN FIRST COMPLETE REMISSION TREATED BY AUTO- OR ALLO-HSCT: RESULTS OF THE PROSPECTIVE GOELAMS LAM-2001 TRIAL

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Background. The prognostic value of NPM1 and FLT3-ITD mutations has now been well established in AML patients with normal karyotype (NK). An international expert panel, on behalf of the European Leukemia Net (ELN), has recently proposed to include NPM1mut/FLT3-ITDwt NK-AML patients in a favorable prognostic subset (Döhner *et al.*, Blood 2010). Autologous hematopoietic stem cell transplantation (auto-HSCT) represented a good alternative therapeutic option for patients without sibling HSC donor. However, the impact of auto-HSCT vs allo-HSCT according to molecular status has been poorly studied. The prognostic impact of the NPM1/FLT3-ITD status was evaluated here in a cohort of 135 NK AML patients prospectively treated by allo-HSCT or auto-HSCT within the randomized GOELAMS LAM-2001 trial (Lioure, ASH 2006, abstracts 319 & 608). *Patients and Methods.* A total of 135 NK-AML patients, aged between 18 and 60 years old (median 47), who had reached a first complete remission (CR) were characterized for NPM1/FLT3-ITD status using previously reported methods (Schnittger *et al.*, Blood 2002 & 2005). According to the ELN classification, the NPM1mut/FLT3-ITDwt status was compared to all other combinations. Randomization allotted these patients to receive an allo-HSCT in case of an available HLA sibling donor while patients lacking such a donor were randomized between one (Arm A) or two (Arm B) auto-HSCT(s). The conditioning regimen was Busulfan + HDM 140 mg/m^2 in Arm A. In Arm B patients received first an auto-HSCT conditioned by HDM 200 mg/m^2 followed by a second auto-HSCT conditioned as for arm A. *Results.* Forty-one patients were programmed to receive an allo-HSCT including 16 with a favorable molecular status, while 94 patients were programmed to receive 1 (n=48) or 2 (n=46) autografts, including 30

with a favorable molecular status. Comparisons of 4-year LFS and OS according to different sub-groups are given in Table 1. No significant difference was observed according to the NPM1/FLT3-ITD molecular status in the group of allo-HSCT patients. Conversely, in the auto-HSCT group, 4-year LFS and OS appeared to be significantly higher in the NPM1mut/FLT3-ITDwt sub-group as compared to other patients (P=0.01 for both). As for the overall auto-HSCT group, no significant benefit of double auto-HSCT vs. single auto-HSCT could be observed regarding each subset of molecular profile. Among the NPM1mut/FLT3-ITDwt subgroup, there was no benefit for allo-HSCT as compared to auto-HSCT. Since there was no significant difference in outcome between allo-HSCT and auto-HSCT patients, the whole cohort was tested for the significance of the molecular status. The statistically significant favorable impact of NPM1mut/FLT3-ITDwt was retrieved in this whole group. *Conclusions.* Molecular analysis of available samples of the GOELAMS LAM-2001 trial of younger AML patients with NK in first CR confirms the favorable prognostic impact of the NPM1mut/FLT3-ITDwt status in patients treated with a single or double auto-HSCT. This procedure represents a valid therapeutic option, providing similar outcomes as allo-HSCT which is not recommended anymore. For patients with other molecular profiles of NPM1/FLT3-ITD, allo-HSCT has to be considered, since this strategy conferred the same outcome as in patients with the favorable mutational status.

Table 1. Outcomes according to different sub-groups.

Normal Karyotype AML	4-year LFS		4-year OS	
Allo-HSCT N=41				
NPM1mut/FLT3-ITDwt N=16	62%		69%	
Other NPM1/FLT3-ITD status N=25	60%	P=NS	55%	P=NS
Auto-HSCT N=94				
1 auto-HSCT N=48	46%		57%	
2 auto-HSCT N=46	41%	P=NS	50%	P=NS
NPM1mut/FLT3-ITDwt N=30	58%		73%	
Other NPM1/FLT3-ITD status N=64	36%	**P=0.01**	44%	**P=0.01**
NPM1mut/FLT3-ITDwt				
1 auto-HSCT N=16	68%		72%	
2 auto-HSCT N=14	49%	P=NS	57%	P=NS
Other NPM1/FLT3-ITD status				
1 auto-HSCT N=32	35%		42%	
2 auto-HSCT N=32	37%	P=NS	47%	P=NS

0033

INDUCTION OF A CD8+ T CELL RESPONSE TO THE MAGE CANCER TESTIS ANTIGEN BY COMBINED TREATMENT WITH AZACITIDINE AND SODIUM VALPROATE IN PATIENTS WITH ACUTE MYELOID LEUKEMIA AND MYELODYSPLASIA

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Background. Epigenetic therapies, including DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors, represent important new treatment modalities in haematological malignancies but their mechanism of action remains unknown. Cancer testis antigens (CTAgs) represent a family of immunodominant proteins normally expressed only in germ line tissue. As a consequence immunological tolerance to CTAgs is not ordinarily established. We and others have demonstrated that the CTAg MAGE is expressed in AML. Since epigenetic therapies result in tumor specific up-regulation of MAGE expression this represent a promising and manipulable tumor antigen in AML. We reasoned that up-regulation of MAGE antigens by DNMT and HDAC inhibitors may induce an immunologically mediated anti-tumor response and contribute to their clinical activity in patients with high risk AML. *Aims.* We therefore characterised the immune response to MAGE antigens in patients with AML undergoing treatment with the epigenetic therapies 5'-azacitidine (AZA) and sodium valproate (VPA). *Methods.* CD8+ T cell responses to MAGE antigens were measured in 21 patients with *de novo* or relapsed AML undergoing treatment on a Phase II clinical trial incorporating AZA (75 mg/m^2 × 7 days per 28 day cycle) and VPA (1-2.5 g daily administered continuously as tolerated). All patients gave informed consent and the clinical trial was approved by the Local Research Ethics Committee. Clinical responses to combined treatment with AZA/VPA were assessed using criteria defined by Cheson. MAGE-specific CD8+ T cells were measured in peripheral blood using a CD137 expression and enrichment assay. In some patients responses were also quantitated by the interferon gamma cytokine secretion assay (Miltenyi Biotec). *Results.* CD8+ CTL responses to MAGE antigens were documented in only 1/21 patients prior to commencement of therapy. 12 patients received at least three cycles of VAL/AZA therapy and were assessable for induction of an immunological response. Of these, treatment with VAL/AZA induced a CD8+ T cell response to MAGE antigens in 10 patients of whom 7 achieved a major clinical response (CR or PR). Responses were maintained over-time (70-799 days) and the CTL response increased in frequency with repeated cycles in the majority of cases. A polyclonal T cell line was generated from one patient and tetramer staining of this line confirmed peptide specificity. The MAGE-specific CTL response detected in two patients was further validated using the IFN-γ cytokine secretion assay. Characterization of the memory phenotype of the CD8+ MAGE-specific response indicated that they were effector memory demonstrating recent T cell activation. Comparison with a control group of 66 patients with high risk AML treated with VAL/AZA demonstrated that generation of a MAGE specific T cell response was associated with a significantly increased chance of achieving a major clinical response (P= 0.038). *Conclusions.* This is the first demonstration of a MAGE specific CTL response in AML. Furthermore, it appears that epigenetic therapies have the capacity to induce a CTL response to MAGE antigens *in vivo* which may contribute to their clinical activity in AML. These data identify a novel mechanism by which epigenetic therapies may induce an anti-tumor response in AML and other malignancies.

0034

DISTINCT CLINICAL AND BIOLOGICAL CHARACTERISTICS OF ADULT PATIENTS WITH DE NOVO ACUTE MYELOID LEUKEMIA WITH ADDITIONAL SEX COMB-LIKE 1 (ASXL1) MUTATION

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Background. Additional sex comb-like 1 (ASXL1) is a member of Enhancer of Trithorax and Polycomb genes and is involved in chromatin modification and retinoic acid pathway regulation. Recent studies showed mutation in this gene in various myeloid malignancies such as myelodysplastic syndrome (MDS), myeloproliferative neoplasm (MPN), and MDS/MPN. However, the ASXL1 mutation in *de novo* acute myeloid leukemia (AML) has not been comprehensively investigated. In this study, we studied this mutation in a large cohort of Chinese primary AML patients. *Aims.* To analyze ASXL1 mutation in adult *de novo* AML patients. *Method*s. We retrospectively analyzed mutation in ASXL1 exon 12 by polymerase chain reaction (PCR) and direct sequencing in 501 adult Chinese AML patients, who were diagnosed at the national Taiwan university hospital from 1995 to 2007. The result was then correlated with the clinical presentations, laboratory data and a panel of 13 important genetic mutations in AML. *Results*. ASXL1 mutations in exon 12, including frame shifting and non-sense mutations, were found in 54 patients (10.8%). The most frequent type is insertion of G between nucleotide 1934 and 1935. The mutation occurred more frequently in elder (P<0.001) and male patients (P=0.016), and was significantly associated with FAB M0 subtype (P=0.003), isolated trisomy 8 (P=0.003), RUNX1 mutation (P<0.001), and expression of HLA-DR (P<0.001) and CD 34 (P=0.020). In contrary, this mutation was inversely associated with NPM1 mutation (P=0.001), FLT3/ITD (P=0.004), and t(15;17) (0/38 vs. 54/425, P=0.029). Among the patients who received standard induction chemotherapy (n=360), those with ASXL1 mutation had lower complete remission rate (85.9% vs 65.2%, P=0.015) and worse overall survival than those without ASXL1 mutation (median overall survival 14 months vs 58 months; P=0.009). This survival difference remained significant when the patients with acute promyelocytic leukemia were excluded (median, 14.0 months vs. 31 months, P=0.023), and became even more pronounced in the subgroup with intermediate cytogenetics (P=0.007). Multivariate analysis including covariates NPM1 mutation, FLT3/ITD, age, and cytogenetics showed a borderline poor prognostic impact of ASXL1 mutation on overall sur-

vival (P=0.049). *Conclusion.* Our study revealed distinct clinical and biological features of *de novo* adult AML patients bearing ASXL1 mutation.

0035

SUPPRESSOR OF CYTOKINE SIGNALING (SOCS1) COOPERATES WITH FLT3-ITD IN THE DEVELOPMENT OF MYELOPROLIFERATIVE DISEASE BY PROMOTING THE ESCAPE FROM EXTERNAL CYTOKINE CONTROL

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Background. Internal tandem duplications of the receptor tyrosine kinase FLT3 (FLT3-ITD) are frequently found in acute myeloid leukaemia and lead to constitutive activation of the receptor. Gene expression analysis comparing cells expressing FLT3-ITD with FL-stimulated cells expressing wild-type FLT3 demonstrated differences in STAT5 target genes. Among the most differentially regulated genes were members of the SOCS (suppressor of cytokine signaling) family, which are known to terminate cytokine signalling by inhibition of JAK kinases. This finding was surprising, as expression of SOCS proteins is associated with anti-proliferative effects. *Aims.* We hypothesised that SOCS proteins function as conditional oncogenes in the context of FLT3-ITD by protecting transformed cells from external cytokine control. The aim of this study was to examine the role of SOCS proteins in FLT3-ITD mediated disease. *Methods.* First, we performed *in vitro* experiments in cell lines and primary AML samples to confirm the microarray data. We performed functional experiments in murine bone marrow cells expressing either SOCS1, FLT3-ITD or both. Finally, *in vivo* bone marrow transplantation experiments were performed to determine the influence on latency and phenotype in this cell system. *Results*. Here we show that FLT3-ITD leads to induction of the suppressors of cytokine signaling (SOCS) family proteins in cell lines, murine bone marrow and patient samples. As SOCS proteins are known to inhibit - rather than promote - growth of hematopoietic cells, we investigated their role in FLT3-ITD mediated transformation. While SOCS1 expression severely impaired cytokine-induced colony growth of murine bone marrow, it failed to inhibit FLT3-ITD supported colony growth, indicating resistance of FLT3-ITD to SOCS1. Also, SOCS1 co-expression did not affect FLT3-ITD mediated signaling or proliferation. Importantly, SOCS1 co-expression inhibited interferon-γ signaling and protected FLT3-ITD cells from interferon-γ mediated growth inhibitory effects. In a murine bone marrow transplantation model, the co-expression of SOCS1 and FLT3-ITD significantly shortened the latency of a myeloproliferative disease when compared to FLT3-ITD (P=0.01). *Summary/Conclusions.* These data demonstrate that in the context of the oncogene FLT3-ITD, SOCS1 acts as a conditional oncogene: expression of SOCS proteins shields FLT3-ITD-expressing cells from external cytokine control, thereby cooperating in the development of a myeloproliferative disease.

0036

THE NON-HOMEODOMAIN SPLICE VARIANT HOXA9T CAUSES LEUKEMIA ON ITS OWN AND COLLABO-RATES WITH HOXA9 TO INDUCE SHORT - LATENCY AML IN MICE

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Background. Alternative splicing is known to affect more than half of all human genes, and has been proposed as a primary driver of the evolution of phenotypic complexity in mammals. Various Hox genes are known to produce alternative transcripts encoding different isoforms whose physiological relevance during development is not yet understood. Hoxa9 is a homeo-box transcription factor with a central role in both hematopoiesis and leukemia. High level of HOXA9 expression is a characteristic feature of acute myeloid leukemia (AML) and is associated with dismal prognosis. Previously a highly conserved alternative splice variant - trun-cated Hoxa9 (Hoxa9T) - has been identified that lacks the homeodomain. So far the biological relevance of Hoxa9T in normal and malignant hematopoiesis is not known. *Aim.* The aim of this project is to identify, characterize and understand the role of the Hoxa9T isoform and Hoxa9 in normal and malignant hematopoiesis. *Methods.* Expression of Hoxa9 and Hoxa9T was analysed in human and murine normal and malignant hematopoiesis by qPCR. To understand the function of the naturally occurring splice variant of HOXA9 we generated two mutants: first, we silenced the pseudo-intron rec-ognition sites of Hoxa9 wild-type to eliminate splicing of Hoxa9 (Hoxa9 FL) and secondly we generated Hoxa9T. *in vivo* function of Hoxa9T was tested in the syngenic murine bone mar-row transplantation model and colony forming unit spleen assay (CFU-S). *Results.* Expression levels of Hoxa9 and Hoxa9T were significantly higher in KSL cells com-pared to normal total BM (>90-fold and >20-fold, respectively). Interestingly, Hoxa9T was higher expressed in murine BM progenitors compared to Hoxa9. HOXA9 and HOXA9T were co-expressed in human leukemic cell lines as well as in 115 AML patient samples with normal karyotype. Interestingly, 90% of the analyzed AML patient samples showed an aberrant ratio between HOXA9 and HOXA9T expression compared to highly purified $CD34^+$ bone marrow cells of healthy donors (ratio=3.2). The vast majority of the AML patients (69%) showed an increased ratio of expression due to an elevated HOXA9- versus HOXA9T-expression, whereas 21% showed a decrease in this ratio. To test the hematopoietic activity of different Hoxa9 iso-forms, we performed CFU-S assay. All the different Hoxa9 forms (Hoxa9WT, Hoxa9T and Hoxa9FL induced an increase in the frequency of short - term repopulating stem cells com-pared to the empty retroviral control in CFU-S assay (n at least12). Importantly, activity of Hox9WT and Hoxa9FL could be further enhanced by co-expression of Hoxa9T. In contrast to the splicable Hoxa9WT (n=21, 135 d), overexpression of Hoxa9T in the BM transplantation model induced AML only after long median latency of 214 days (n=21). Also the non-spliced isoform Hoxa9FL did show a delay in median latency compared to Hoxa9WT (median latency 208 vs. 135 days). To confirm the hypothesis that this delay is due to a loss of Hoxa9T, we co-expressed Hoxa9T and Hoxa9FL (n=10) which again led to a median latency of 144 days comparable to Hoxa9WT. *Conclusions.* These results indicate that that the leukemogenic po-tential of Hoxa9 depends on the collaboration with its own spliced isoform

0037

HIGH-RESOLUTION MOLECULAR ALLELOKARYOTYPING IDENTIFIES NOVEL UNIPARENTAL DISOMY AND FOCAL COPY NUMBER ALTERATIONS IN ACUTE PROMYELOCYTIC LEUKEMIA (APL)

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Introduction. Experimental evidence obtained in transgenic mice revealed that PML-RARA is necessary but not sufficient for the development of APL, suggesting that additional genetic mutations are also required for its development. *Aim.* To define whether additional submicroscopic genomic alterations may characterize APL and may be used to better classify the disease by dissection of genomic subsets. *Patients and Methods.* 105 adult patients with acute myeloid leukemia were analyzed. These cases included all French-American-British subtypes, miscellaneous cytogenetic abnormalities and normal karyotype subgroups. Among these, the M3 subtype included 28 patients, representing the 33% of the whole study population. Genomic DNA was isolated from blast cells and applied to Genome-Wide Human SNP 6.0 array (Affymetrix, Santa Clara, CA) following the manufacturer's instructions. Fluorescence *in situ* hybridization, quantitative PCR and nucleotide sequencing were used to confirm genomic alterations. *Results.* A wide spectrum of different copy number alterations (CNAs) were identified in all cases and no significant difference in the average number of alterations was detected among different leukemia cytogenetic subgroups except for the complex subgroup, which had an average of 55 CNA/patient. In APL cases an average of 8 CNAs per case (range, 1-24) was found. The macroscopic alterations were rare, confirmed conventional cytogenetics and involved trisomy of chromosome 8 in 3 cases, loss of chromosome 6, loss of chromosome 20 and deletions on chromosome 9 and 7. Microscopic CNAs (<1.5 Mbps) involved every chromosome at least once and predominantly chromosomes 1, 2, 9, 15 and 17. For each alteration we interrogated a collated library of copy-number variants (CNVs, Database of Genomics Variants and USCS Genome Browser) to assure that these regions were not known as CNVs and therefore to decrease the noise of raw copy number data.

Genetic gains were more common than losses and their median size was 300 kb (range 0.2- 1.4 Mb). The majority of lesions were not recurrent, being identified in only a single patient. Focal genetic alterations were detected at the breakpoints of t(15;17)(q22;q21) in PML and RARA genes, in genes involved in activation of transcription (loss of LMX1 on 1q23.3, loss of MLXIPL and BCL7 on 7q11.23), regulation of cell cycle (gain of PVT1 and MYC on 8q24) and cell adhesion (gain of NCAM1 on 11q23). In order to identify potential pathogenetic alterations, all microscopic CNAs were compared with the list of genes from the Cancer genome project (http://www.sanger.ac.uk/genetics/CPG/Census) finding out that six alterations involved a known cancer-related gene. Most of these genes encode tyrosine kinase proteins (ERBB4) or transcription factors (ETV1, ETV6, ERG). Copy neutral loss of heterozygosity events affected 1p34.2-1p32.3, 10p11.2 (MLLT10), 11p11.2 (WT1, CDKN1C, HRAS). Finally, patients with more than 10 CNAs were found to be associated with a worse prognosis. *Conclusions.* These data demonstrate that different cooperating events may be involved in the generation of APL. Furthermore, these novel findings may be used to stratify patients according to genomic changes. Supported by: European LeukemiaNet, AIL, AIRC, Fondazione Del Monte di Bologna e Ravenna, FIRB 2006, Ateneo RFO grants.

0038

CLINICAL IMPLICATIONS OF A SPECIFIC METHYLATION PROFILE IN ACUTE MYELOID LEUKEMIA

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Background. Several reports have identified a growing number of genes that may be inactivated due to aberrant hypermethylation in acute myeloid leukemia (AML). However, in most cases the methylation status has been investigated in small number of AML patients and, therefore, its clinical impact has not been yet clarified. *Objectives*. To analyze the clinical relevance of aberrant methylation in a panel of key cancer genes: p15INK4B, RARB, p73, APAF1, SHP1, CDH1 and BLU in a large series with *de novo* AML. These genes were selected based on previous reports where aberrant methylated genes in AML were identified. *Methods.* Bone marrow samples from 184 AML patients at diagnosis were analyzed (110 male/74 female; median age: 60 yr, range: 16-92). Genomic DNA was extracted using standard protocols. After bisulphite treatment DNA was amplified with Methylation Specific Polymerase Chain Reaction using primers specific to the methylated and unmethylated alleles of the genes. *Results.* The methylation frequencies were as follows: 59% p15INK4B, 43% BLU, 42% CDH1, 41% p73, 33% SHP1, 30% RARB and 22% APAF1. A total of 163 patients (88%) had at least one gene methylated. Only three patients were found to have concurrent methylation of the seven genes studied. Aberrant methylation of any individual gene was compared with patient characteristics, including: age, sex, WBC count, FAB subtype, cytogenetic risk groups and FLT3-ITD mutations. Methylation of p15INK4B and RARB were associated with a leukocyte count ≤10×10^9/L (P=0.04), while methylation of p73 was associated with age ≤60 years (P=0.04) and leukocyte count ≤ 10×10^9/L (P= 0.02). In univariate analysis, methylation of p15INK4B and BLU were found to have a negative impact in both overall survival (OS) and relapse-free survival (RFS). Therefore, we classified AML patients into two methylation groups according to the presence of concurrent methylation of both p15INK4B and BLU, irrespective of the other genes methylated, (Group A) and the rest of patients (Group B). In univariate analysis, patients belonging to the Group A had a significantly reduced OS than Group B at 4 years (10% versus 28%, respectively, P=0.005), disease-free survival (DFS) (25% versus 6%, respectively, P=0.009) and RFS (28% versus 47%, respectively, P=0.04). In multivariate analysis, the concurrent methylation in both p15INK4 and BLU genes retained an independent adverse significance for OS, DFS and RFS. Similar results were obtained when the analysis was restricted to patients younger than 60 years with intermediate risk cytogenetics. Patients belonging to Group A had a significantly reduced DFS than Group B at 4 years (23% versus 48%, respectively, P=0.03) and RFS (27% versus 66%, respectively, P=0.003). *Conclusions.* Our results indicate that simultaneous aberrant methylation affecting key cancer genes is a common phenomenon in AML. Moreover, the concurrent methylation of p15INK4 and BLU seems to be an important factor in predicting the clinical outcome of AML patients.

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0039

IL-12 DEBULKS PEDIATRIC ACUTE MYELOID LEUKEMIA CELLS BY ACTING ON TUMOR INITIATING CELLS IN VIVO

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Background. Acute myeloid leukemia (AML) is a hematologic tumor which represents 15-20% of all childhood acute leukemias and is responsible for more than half of leukemic death in these patients who frequently relapse. The bulk tumor is continuously regenerated and sustained by rare tumor initiating cells (TIC), that proliferate slowly thus resulting refractory to chemotherapeutic agents that target highly proliferating cells within the tumor. Therefore, complete eradication of the bulk tumor may depend on efficacy of therapies that target TIC. IL-12 is an immunomodulatory cytokine which functions as anti-tumor agent in solid and hematologic malignancies through direct effects on tumor cells, stimulation of the immune system and anti-angiogenic mechanisms. The ability of IL-12 to target the bulk AML cells has never been investigated. *Aims.* Therefore, we asked whether this cytokine may affect *in vivo* blast AML cells and TIC compartment. *Methods.* First, the expression of both chains of the IL-12 receptor was investigated, by flow cytometry, in TIC and in blast AML cells, obtained from pediatric patients at diagnosis (n=16). Next, the *in vivo* IL-12 anti-tumor activity was evaluated by sub-cutaneous (s.c) and intra-venous (i.v.) inoculation of primary AML cells into SCID/NOD Il2rg-/- (NOG) mice that were treated with human recombinant (hr)IL-12 or PBS (controls). AML cells were analyzed at the primary site of tumor cell inoculation, in the peripheral blood and lymphoid organs (e.g. bone marrow and spleen) using human cell surface markers and flow cytometry analysis and/or immunohistochemistry. The IL-12 anti-tumor activity *in vivo* was tested in terms of inhibition of tumor mass formation, cell spreading and angiogenesis. *Results.* We found that i) primary blast AML cells and TIC express both chains of the IL-12R at surface level, ii) AML cells injected into NOG mice displayed enriched TIC population as compared to the original AML cell sample, iii) AML cells injected s.c. into NOG mice gave rise to a tumor mass including TIC at the primary site of cell inoculation. TIC and bulk tumor were eliminated by hrIL-12 treatment, iv) hrIL-12 treatment hindered AML cell homing to the spleen and bone marrow once injected i.v., v) TIC were virtually absent in spleens and bone marrow from NOG mice inoculated with AML cells and subsequently treated with hrIL-12, and viii) IL-12 reduced primary AML angiogenesis *in vitro*. *Conclusion.* The novelty of these results is manifold and may be deals with the demonstration that IL-12 may target blast AML cells and TIC *in vivo*.

0040

THE VENT-LIKE HOMEOBOX GENE VENTX IS A NOVEL HUMAN HEMATOPOIETIC FACTOR, WHICH PROMOTES MYELOID DEVELOPMENT AND IS HIGHLY EXPRESSED IN ACUTE MYELOID LEUKEMIA

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Background. Recent studies suggest that a variety of regulatory molecules active in embryonic development such as clustered and non-clustered homeobox genes play an important role in normal and malignant hematopoiesis. Since it was shown that the Xvent-2 homeobox gene is part of the BMP-4 signalling pathway in Xenopus, it is of particular interest to analyse the function of its only recently discovered human homologue VENTX in normal hematopoietic and leukemic development. *Aim.* The aim of this project is to identify, characterize and understand the role of the Vent-like homeobox gene VENTX in normal and malignant haematopoiesis. *Methods.* Expression of the VENTX gene was analysed in human normal and malignant haematopoiesis by microarray and qPCR. To test the impact of VENTX2 overexpression on human clonogenic progenitor cells, $CD34^+$ cord blood (CB) cells

were retrovirally transduced with VENTX or empty vector and we performed *in vitro* and *in vivo* assays. *Results.* So far we and others have not been able to identify a murine Xenopus xvent gene homologue. However, we were able to document the expression of this gene by qPCR in human lineage positive hematopoietic subpopulations. Amongst committed progenitors VENTX was significantly 13-fold higher expressed in $CD33^+$ BM myeloid cells (4/4 positive) compared to $CD19^+$ BM lymphoid cells (5/7 positive, P=0.01). Of note, expression of VENTX was negligible in normal $CD34^+/CD38^-$ but detectable in $CD34^+$ BM human progenitor cells. In contrast to this, leukemic $CD34^+/CD38^-$ from AML patients (n=3) with translocation t(8,21) showed significantly elevated expression levels compared to normal $CD34^+$ BM cells (n=5) (50-fold higher; P≤0.0001). Furthermore, patients with normal karyotype NPM1c+/FLT3-LM- (n=9), NPM1c-/FLT3-LM^+ (n=8) or patients with t(8;21) (n=9) had an >100 fold higher expression of VENTX compared to normal $CD34^+$ BM cells and a 5- to 7.8-fold higher expression compared to BM MNCs. Gene expression and pathway analysis demonstrated that in normal $CD34^+$ cells enforced expression of VENTX initiates genes associated with myeloid development and downregulates genes involved in early lymphoid development. Functional analyses confirmed that aberrant expression of VENTX in normal $CD34^+$ human progenitor cells induced a significant increase in the number of myeloid colonies compared to the GFP control with 48±6.5 compared to 28.9±4.8 CFU-G per 1000 initially plated $CD34^+$ cells (n=11; P=0.03) and complete block in erythroid colony formation with an 81% reduction of the number of BFU-E compared to the control (n=11; P<0.003). In a feeder dependent co-culture system, VENTX impaired the development of B-lymphoid cells. In the NOD/SCID xenograft model, VENTX expression in $CD34^+$ CB cells promoted generation of myeloid cells with an over 5-fold and 2.5-fold increase in the proportion of human $CD15^+$ and $CD33^+$ primitive myeloid cells compared to the GFP control (n=5, P=0.01). Summary: Overexpression of VENTX perturbs normal hematopoietic development, promotes generation of myeloid cells and impairs generation of lymphoid cells *in vitro* and *in vivo*. Taken together, these data extend our insights into the function of human embryonic mesodermal factors in human hematopoiesis and indicate a role of VENTX in normal and malignant myelopoiesis.

0041

BIOLOGICAL CHARACTERIZATION OF AML AT THE SINGLE CELL LEVEL BASED ON RESPONSE TO DNA DAMAGING AGENTS

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Background. Standard approaches to treating AML are predominantly based on DNA damaging agents. Yet the long-term outcomes of these patients continue to be sub-optimal, most likely due to the cytogenetic and molecular heterogeneity of the disease. We hypothesized that this heterogeneity is reducible to a limited number of intracellular signaling phenotypes that can classify the disease into biologic, mechanistic and clinical subgroups relevant to disease management. A very relevant technology for evaluating these responses is single cell network profiling (SCNP) utilizing flow cytometry which differs from most proteomic technologies by measuring multiple modulated signaling responses at the single cell level and in a manner associated with cell type and other co-expressed proteins. *Aims.* In this study SCNP was utilized to define myeloid growth factor and cytokine-mediated intracellular signaling responses in AML samples and how they relate to *in vitro* responsiveness to DNA damaging agents currently used in therapeutic regimens. Identification of pathways associated with responsiveness or refractoriness to DNA damaging agents may inform the choice of specific therapeutic regimens. *Methods.* Modulated Jak/Stat and PI3K pathway activity was measured after exposure of 34 diagnostic non-M3 AML blasts to myeloid growth factors (e.g Flt3L, SCF), cytokines (e.g G-CSF, GM-CSF) and interleukins (e.g IL-6, IL-27). DNA damage response (DDR) and apoptosis pathway activity of AML blasts exposed to etoposide was determined by measuring levels of p-Chk2 and cleaved PARP respectively. Samples were processed for cytometry by paraformaldehyde/methanol fixation and permeabilzation followed by incubation with panels of fluorochrome-conjugated antibodies that recognize cell surface proteins to delineate cell subsets and intracellular pathway molecules. *Results.* Analysis of modulated Jak/Stat and PI3K pathway responses in individual patient samples identified blast subgroups with distinct pathway profiles: A) high Jak/Stat activity B) high PI3K activity C) high activity in both pathways D) low activity in both pathways. *in vitro* exposure of samples to etoposide revealed three distinct "DDR/apoptosis"profiles: 1) AML blasts with a defective DDR and failure to undergo apoptosis 2) AML blasts with proficient DDR and failure to undergo apoptosis 3) AML blasts with proficiency in both DDR and apoptosis. AML samples from clinical responders fell within the third "DDR/apoptosis"profile and had low PI3K and Jak/Stat signaling responses. However, AML blasts from clinically non-responsive cases fell within all the Jak/Stat and PI3K pathway profiles A-D and within all the "DDR/apoptosis"profiles 1-3. Notably, analysis of Jak/Stat, PI3K and apoptosis pathway responses characterized biologically distinct patient-specific profiles of chemo-resistance, even within cytogenetically and phenotypically uniform patient subgroups. *Conclusions.* SCNP revealed cell subsets with distinct signaling, DDR and apoptosis responses between AML samples. The data from this study generated the hypothesis that the requirements for chemo-sensitivity are very restricted: low PI3K and Jak/Stat signaling and proficient DDR and apoptosis. By contrast many pathway aberrations could result in refractoriness to chemotherapy. Further dissection of these and additional pathways in independent AML sample cohorts could provide guidance for the most appropriate choice of therapy in individual patients.

0042

A HIGH AMOUNT OF $CD34^+$ CD38LOW/NEG $CD123^+$ CELLS HAS AN ADVERSE IMPACT ON DISEASE FREE AND OVERALL SURVIVAL IN AML PATIENTS TREATED BY INTENSIVE CHEMOTHERAPY

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Background. The characterization of new prognosis tools is of major importance in acute myeloid leukemia (AML). The high expression of CD123 (the IL3 alpha chain receptor/IL3-R) on myeloid blast cells was previously reported as a marker of adverse outcome in AML and anti-IL3-R therapies are currently in development and may be useful to prevent AML relapses. The $CD34^+$ $CD38^{low/neg}$ $CD123^+$ phenotype identifies a subset of AML cells enriched in leukemic stem cells. *Aims.* The aim of the study was to evaluate the prognostic impact of the amount of $CD34^+$ $CD38^{low/neg}$ $CD123^+$ cells detected at diagnosis among the AML bulk. *Patients and Methods.* Quantification of blast cells with the CD34+ CD38low/neg $CD123^+$ phenotype was achieved by flow cytometry in 144 patients with *de novo* AML (median age 56 y). Distribution among cytogenetic groups was: unfavourable 15%, intermediate 65% and favourable 19%. The survival studies were performed for 116 CD34 positive patients homogeneously treated according to intensive chemotherapy trials from the French GOELAMS group. Informed consent was obtained in accordance to the declaration of Helsinki. *Results.* The percentage of $CD34^+$ $CD38^{low/neg}$ $CD123^+$ cells (LSC%) is highly variable between samples (0-67%, median: 2.7%) and appeared independent from the FAB classification, the age or the cytogenetic group. The LSC% has no impact on the achievement of complete remission (CR=85%) among the 116 patients treated with intensive chemotherapy but is higher in patients who died (3.7 vs 0.6%, P=0.003) or relapsed (4.3 vs 0.7%, P=0.02). With a 1% cut-off, LSC% is tightly correlated with overall survival (OS), with a median survival of 32 months versus 16 months (P=0.005). Similarly, the disease free survival (DFS) is 37 vs 11 months in the 91 patients who achieved complete remission (CR) (P=0.001). Considering the intermediate cytogenetic group (n=75), the LSC% is also predictive for a better OS (32 vs 16 months, P=0.017) and DFS (37 vs 14 months, P=0.01), even in the Flt3 wild type subgroup (OS: 78 vs 26 months, P=0.01). Interestingly, in CBF leukemias (n=25), the LSC% is still predictive for a better DFS (36 vs 13 months, P=0.02). *Conclusion.* This study emphasizes the prognosis impact of the $CD34^+$ $CD38^{low/neg}$ $CD123^+$ cell burden in AML patients, which is predictive of shorter OS and DFS when representing over 1% of the leukemic cells, regardless of the usual prognosis categories. We provide here a new prognosis marker that may be easily translated to the clinical practice in AML although it remains to be validated on a large prospective cohort of patients. Moreover, new therapies targeting this subpopulation could help to improve outcome in AML patients.

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0043

THE MOLECULAR PATHWAYS AFFECTED BY THE AML ASSOCIATED ONCOFUSION PROTEINS AML1-ETO AND PML-RAR

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Background. Recurring chromosomal abnormalities have been identified in a variety of cancers, but are most frequently associated with hematological malignancies such as acute myeloid leukemia (AML) and acute lymphocytic leukemia (ALL). At present, more than 1000 recurring cytogenetic abnormalities have been reported for AML alone. Three main cytogenetic changes have been detected in leukemic cells: chromosomal deletions, inversions and translocations, with translocations being by far the most frequent. In addition to transcriptional activation of proto-oncogenes these cytogenetic changes often cause gene fusions. In these cases the translocation splits the genes on both partner chromosomes leading to the juxtaposition of part of each gene. The resulting fusion gene often encodes a chimeric protein. Although many of the breakpoints involved in specific chromosomal translocations have been cloned, in most cases the role of the chimeric oncofusion proteins in tumorigenesis is not elucidated. *Aim.* A rational approach towards the definition of specific treatment for AML requires identification of the molecular mechanisms that are utilized to transform hematopoietic progenitors. For this, we wished to characterize the genome-wide binding profiles of the fusion products of 2 translocations that are frequently associated with AML: t(8;21) and t(15;17) and characterize their common gene programs and associated pathways. *Methods.* Both cell lines and patient blasts expressing either PML-RAR or AML1-ETO were subjected to chromatin immunoprecipitation experiments using sets of antibodies that could recognize different epitopes within the oncofusion proteins. Precipitated DNA was subsequently analyzed with large-scale sequencing techniques allowing the identification of the global binding patterns of the chimeric proteins. *Results.* Comparison of AML1-ETO and PML-RAR target regions revealed many common target genes, amongst which the hematopoietic master regulator SPI1/PU.1. In addition, bioinformatic analysis of the common binding sites revealed that both proteins target various signaling pathways. Finally, genome-wide epigenetic studies revealed decreased histone acetylation upon binding of both proteins, suggesting the involvement of the oncofusion proteins in the recruitment of enzymes that modulate histone acetylation levels. *Conclusions.* Together, these results suggest that the molecular mechanisms AML1-ETO and PML-RAR use to transform hematopoietic progenitors significantly overlap. The results suggest that both proteins utilize the recruitment of histone deactylases to modulate expression of genes important for hematopoietic differentiation and for various signaling pathways.

0044

TARGETED INHIBITION OF MTOR BY AZD8055 BLOCKS PROTEIN TRANSLATION AND HAS ANTI-LEUKEMIC ACTIVITY IN ACUTE MYELOID LEUKEMIA

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Background. The treatment of acute myeloid leukemia (AML) still represents a challenge. Major efforts are therefore made to develop targeted molecules against deregulated signaling pathways that sustain AML cells growth and survival. The mTOR serine/threonine kinase belongs to two separate complexes, mTORC1 and mTORC2. The mTORC1 complex, which is inhibited by rapamycin in most models, controls mRNA translation through the phosphorylation of the translation repressor 4E-BP1. The frequent activation of mTORC1 in primary AML cells underscored this complex as a major target for AML therapy. However, the allosteric inhibition of mTORC1 by rapamycin has only modest effects in AML. The rather low anti-leukemic activity of rapamycin has already been linked to different resistance mechanisms. We recently showed that the translation process is deregulated and resistant to rapamycin in AML. Moreover, the mTORC2 complex, which is responsible for the full activation of the oncogenic kinase Akt through its PDK2 activity, is generally reported as resistant to rapamycin. Finally, mTORC1 inhibition by rapamycin induces a positive feedback on the PI3K/Akt signaling involving mTORC2 activity. The AZD8055 compound (AstraZeneca, UK) is an ATP-competitive inhibitor specific of the mTOR kinase. We thus hypothesized that direct inhibition of the catalytic activity of mTOR by AZD8055 could overcome the rapamycin resistance phenotype in AML. *Materials and Methods.* AZD8055 was used from 1 nM to 1000 nM. The anti-leukemic activity of AZD8055 was tested in both human AML cell lines (MV4-11, MOLM-14, OCI-AML3) and in 20 primary AML samples all included in trials initiated by the French GOELAMS group. Protein translation was tested by 7Methyl Guanosine cap affinity assay, polysomes analysis and [3H]Leucine incorporation assay. Blast cell proliferation and apoptosis were quantified by [3H]Thymidine incorporation and annexin-V staining, respectively. *Results.* We first observed that in contrast to rapamycin, AZD8055 dose-dependently inhibits mTOR catalytic activity, attested by the inhibition of mTOR S2481 phosphorylation. Accordingly, AZD8055 blocks mTORC1 activity, attested by the decrease of P70S6K T389 phosphorylation and also mTORC2 activity, as shown by the inhibition of Akt S473 and of NDRG1 T346 phosphorylations. Furthermore, AZD8055 inhibits the rapamycin-resistant phosphorylation of the translation repressor 4E-BP1 on T37/46, T70 and S65 residues. This results in a marked inhibition of mRNA translation in AML cells, attested by (i) an inhibition of the assembly of the translation initiating complex eIF4F, (ii) a shift from large to small polysomes in AZD8055-treated AML cells and (iii) a decreased expression of the oncogenic cap-dependant proteins Bcl-xL, c-Myc and cyclinD1. Accordingly, AZD8055 has a marked anti-leukemic activity in AML. Indeed, it strongly reduces the proliferation of AML cells and blocks the cell-cycle progression. AZD8055 also represses the clonogenic growth of AML progenitors and induces in the killing of AML blast cells, but not of normal $CD34^+$ hematopoietic cells. *Conclusion.* AZD8055 compound demonstrates a remarkable anti-leukemic activity through specific inhibition of both mTORC1 and mTORC2 activities in AML cells. The absence of toxicity against normal hematopoietic cells ex vivo highly suggests a favourable therapeutic index, which emphasize the development of AZD8055 as a clinical candidate for therapy in AML.

0045

TARGETING HEMATOPOIETIC STEM CELLS EXPANSION IN C/EBPALPHA MUTANT AML

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Background. Acquisition of oncogenic mutations in stem cells is an attractive model for multistep tumorigenesis in highly self-renewing tissues. We therefore demonstrated that acute myeloid leukemia (AML) is a multistep disease with premalignant hematopoietic stem cells (HSC) expansion preceding the formation of leukemia initiating cells (LIC) with committed myeloid phenotype. Patient-derived C-terminal C/EBPalpha mutations increase the proliferation of long-term hematopoietic stem cells (LT-HSCs) in a cell-intrinsic manner and override normal HSC homeostasis, leading to expansion of premalignant HSCs. Such mutations impair myeloid programming of HSCs and block myeloid lineage commitment when homozygous. N-terminal C/EBPalpha mutations instead are silent with regards to HSC expansion, but allow the formation of committed myeloid progenitors, the templates for LICs. The combination of N- and C-terminal C/EBPalpha mutations incorporates both features, leading to accelerated AML development in mice, thus explaining the most prevalent C/EBPalpha mutation pattern in AML patients. *Aims*. Since HSC expansion in biallelic C/EBPalpha mutant AML is associated with accelerated disease, these expanding HSCs represent a novel target for cancer therapy, distinct from successively generated LICs. We would like to find genetic and pharmacologic ways to impede abberrant mutant HSC expansion. Cell cycle analysis have demonstrated that all mutant HSC compartments including LT-HSCs have increased cycling, suggesting that they can be targeted by antiproliferative drugs used in conventional chemotherapy. We use cytosine arabinoside (Ara-C) to check whether 1) it leads to selective mutant HSCs apoptosis and consequent drop in cell number; 2) whether it affects long-term survival of leukemic mice. *Methods.* We

use recently described mouse model of biallelic C/EBPalpha mutant AML. We perform Ara-C treatments in radiation chimeras competitively transplanted with a mix of wild type and C/EBPalpha mutant fetal liver cells. This way the effect of chemotherapy is evaluated on both wild-type and mutant HSCs in the same mouse. Apoptosis in wild type (CD45.1+) and mutant (CD45.2+) LT-HSCs is measured by fluocytometry (LT-HSCs are defined as Lin-c-kit+Sca-1+CD150+) using AnexinV/SytoxBlue staining. Frequency and total number of HSCs is calculated in mice after 24 and 72 hours post-treatment. *Results*. We found that Ara-C efficiently and selectively induced apoptosis in mutant HSCs and downregulated their frequency and total number. However it did not lead to their complete elimination. Future studies will focus on characterization of the Ara-C resistant cells to find possible combination treatment strategies to block HSC expansion and slow down or prevent leukemia progression. *Summary*. This study provides proof of principle that hyper-proliferating mutant LT-HSCs can be selectively targeted by antiproliferative agents. Future work will focus on characterization of therapeutic relevance of the described results and finding complementary pathways that would allow elimination or proliferation block of the residual leukemogenic HSCs.

0046

TARGETING IRON HOMEOSTASIS INDUCES CELLULAR DIFFERENTIATION AND SYNERGIZES WITH DIFFERENTIATING AGENTS IN ACUTE MYELOID LEUKEMIA

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Background. Differentiating agents have been proposed to overcome the impaired cellular differentiation in acute myeloid leukemia (AML). However, only the combinations of all-trans retinoic acid or arsenic trioxide with chemotherapy have been successful, and only in treating acute promyelocytic leukemia. Iron is required as a cofactor for a number of critical cellular enzymes involved in energy metabolism and cell proliferation and therefore is essential for all living cells. Iron chelators represent an alternative anti-cancer therapy and effectively induce cell growth arrest and apoptosis in prostate and melanoma cancer cells *in vitro* and *in vivo*. *Aims.* We aimed to characterize the molecular mechanisms underlying the anti-tumor effect of iron-chelating therapeutic approaches and to validate their potential value in AML therapy. *Methods*. We worked with HL60, OCI-AML3, THP1, U937 and NB4 leukemia cell lines and peripheral blood cells issued from AML patients and healthy donors after obtaining their written informed consent. Cell differentiation was assessed by CD14 and CD11b expression and esterases activity. Fluorescence analysis was used to determine the relative levels of reactive oxygen species (ROS). Mitogen-activated protein kinases (MAPKinases) and Vitamin D (VD) pathways were studied by RQ-PCR and western blots. *in vitro* observations were confirmed in a nude mice model subcutaneous xenografted with HL60 and OCI-AML3 cell lines. *Results.* Here we showed that iron homeostasis is an effective target in the treatment of AML. Iron chelating therapy induces the differentiation of leukemia blasts and normal bone marrow precursors into monocytes/macrophages in a manner involving modulation of ROS expression and the activation of MAPKinases. By comparing the pattern of genes induced by VD and iron-chelating agents in a non-supervised transcriptome analysis, we found that thirty percent of the genes most strongly induced by iron deprivation are also targeted by VD, a well-known differentiating agent. Iron chelating agents induce expression and phosphorylation of the VD receptor, and iron deprivation and VD act synergistically. VD magnifies activation of MAPK JNK and the induction of VD receptor target genes. The efficacy of differentiation therapy was further evaluated *in vivo* in a mouse tumor xenograft model. The combination of VD with DFO significantly reduced tumor growth. When used to treat one AML patient refractory to chemotherapy, the combination of iron chelating agents and VD resulted in reversal of pancytopenia and blast differentiation. *Conclusions.* We propose that iron availability modulates myeloid cell commitment, and that targeting this cellular differentiation pathway together with conventional differentiating agents provides new therapeutic modalities for AML. This combined therapy would be particularly useful in elderly patients who are not eligible for high dose chemotherapy and bone marrow transplantation. This association could be also relevant in other diseases involving a deregulation of BM differentiation, such as myelodysplastic syndromes. Additional clinical studies are needed to validate its efficacy.

0047

MONITORING OF ABERRANTLY METHYLATED GENES IN AML PATIENTS

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Changes in methylation pattern are well known to be involved in pathogenesis of patients with acute myeloid leukemia (AML). Aberrant methylation pattern has been described as a potential biomarker for cancer cells detection. The aim of the current study was to introduce quantitative methylation specific real-time PCR based method called MethyLight for routine monitoring of disease progression by quantifying the level of methylation. We also searched for novel genes influenced by DNA methylation. To evaluate DNA methylation levels of patients with AML, 52 AML samples at diagnosis and 196 at remission were examined. Median follow-up after diagnosis was 16 months (range 2-30) Genes previously described as often affected by hypermethylation either in hematological or other malignancies were chosen. These genes included: CDKN2B, ESR1, ECAD, DAPK1, CALCA, ICAM1, MYOD1, SOCS1, TIMP-3 and TERT. 100% methylated DNA was utilized as a reference to which methylation levels were expressed as a percentage of methylated reference (PMR). We also searched for novel genes affected by DNA hypermethylation. ML-2 cell line were treated for 3 days with 1 μM decitabine alone, 0.1 μM TSA alone or decitabine plus TSA, followed by Human Signal Transduction Pathway Finder PCR expression array. We confirmed high methylation frequency of studied genes (75%) in 52 AML patients at diagnosis. The most frequently hypermethylated genes were the following: CDKN2B (71%), ECAD (63%), CALCA (50%) and MYOD1 (33%). All remission samples showed negativity for methylation, which means they were under the level of methylation found in normal peripheral blood cells. Reoccurrence of DNA hypermethylation in AML patients with at least one hypermethylated genes at diagnosis were observed in disease relapse (5/5). After cultivation of ML2 cell line about 15 genes were significantly upregulated by twofold or greater when comparing to the control without hypometylating agents. Comparison of gene expression patterns in the treated versus control cells has revealed cohorts of genes that play role in differentiation (EGR1, CEBPB, BMP2), activation of immune system (CCL2, CCL20, IL8, IL4R, ICAM1), apoptosis (BAX, IL1A, LTA, GADD45A) and cell cycle regulation (CDKN1A, CDKN2B). The EGR1 gene was of particular interest, because it has been desribed as a potential tumour suppressor gene in hematological malignancies. We verified changes in EGR1 expression by performing TaqMan RQ-RT PCR not only in cultivated cells but also in AML patients at diagnosis versus healthy donors. A substantial proportion of AML patients were noted to have decreased level of EGR1 expression. To clarify the correlation between EGR1 expression and methylation, bisulfite sequencing was performed. It showed no correlation between changes in EGR1 expresion and aberrant DNA promoter methylation. Our data demonstrate the utilization of methylation biomarkers for monitoring minimal residual disease in AML patients and the clear connection between reoccurrence of DNA hypermethylation and disease reoccurrence. Further studies on the rest of upregulated genes after decitabine and TSA exposure are ongoing.

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0048

SNP ARRAYS ANALYSIS REVEALS FREQUENT AND RECURRENT ACQUIRED GENOMIC ALTERATIONS IN AML COPY NUMBER VARIATIONS OR UNIPARENTAL DISOMY (A STUDY OF THE ALFA GROUP)

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Introduction. Acute myeloid leukemia (AML) is a heterogeneous group of pathology with variable response to treatment. In addition to the age and leukocytosis at diagnosis, cytogenetic abnormalities are key factors to assess prognosis. Although the identification of alteration affecting *CEBPa*, *NPM1* and *FTL3* improved the characterization of

AML, the variability can not be completely explained and several genomic alterations are likely to be discovered. In this study, we performed genomic profiling on a cohort of 130 AML patients in order to identify new genomic alterations and determine candidate gene potentially involved in oncogenesis or tumoral progression. *Methods.* This study was conducted on a cohort of 130 AML patients who have reached complete remission (CR). Cases were classified according to MRC cytogenetic classification (28 favourable; 85 intermediates, 7 adverse, 10 without any informative karyotype). Patients were aged from 3 to 65. Cases were distributed across all French-American-British (FAB) classes except M3 (8 M0, 25 M1, 39 M2, 26 M4 and 11 M4Eo, 12 M5, 2 M6, 1 M7), In all patients, DNA was extracted from bone marrow sample obtained at diagnosis and after CR achievement. Paired diagnosis and CR DNAs were analyzed using Affymetrix SNP Array 6.0 in order to distinguish acquired from constitutional anomalies. Acquired Copy number variations (CNA) were validated on a custom Agilent microarray 105k. *Results.* In this cohort, we found 209 genomic abnormalities in 73 patients (56%): 197 CNA and 13 copy neutral losses of heterozygoty (partial uniparental disomy or UPD). Each patient had 0 to 16 anomalies (median=1). Among CNA, deletions were more frequent than gains (130 vs 66). CNA spanned from 8 kb to 191 MB (median of gains 24 MB, median of losses 2 MB). 116 of them had not been detected by conventional cytogenetics. UPD spanned from 23 MB to 150 MB (median, 33 MB). In our cohort, anomalies were located over all chromosomes except chromosome 14 and were particularly frequent on chromosomes 2, 7, 11, 16, 17, and 21 (54% of all anomalies). We defined 72 minimal common regions which were altered in at least 2 patients. Among the 43 common regions shorter than 5 Mb, 16 contain at least one gene reported in AML or cancer: Among the 73 patients with CNA or UPD, 17 had a normal karyotype (30% of patients with normal cytogenetics). Frequency of CEBPa and FLT3 mutations (*FLT3*-TKD) was not significantly different in comparison to patients without any CNA or UPD. However, *FLT3* duplication (*FLT3*-ITD) and NPM1 mutations were significantly associated with the absence of CNA or UPD (respectively P=0.01 and P<0.001). This lower frequency of *NPM1* mutations was still significant within the intermediate cytogenetic subclass (P<0.001). *Conclusions.* In our cohort, CNA or UPD was detected in 56% of patients, frequently altering gene previously reported in oncogenesis. These alterations were associated with a specific genotype. In association with conventional cytogenetic, microarray Genomic profiling could better define prognosis subgroup

0049

ACCUMULATION OF 2-HYDROXYGLUTARATE (2-HG) IN NORMAL KARYOTYPE AML PATIENTS WITH IDH1 MUTATION

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Background. Heterozygous mutations in isocitrate dehydrogenase 1 (*IDH1*) were recently identified in AML using massively parallel DNA sequencing. These mutations occur in ~8% of AMLs and appear to have a higher frequency in normal karyotype patients. The *IDH1* gene is a major mutational target in brain cancer, where the mutation leads to a gain-of-function. This results in the production and accumulation of 2-HG from the normal IDH1 metabolic product α-ketoglutarate, produced by the unaffected copy of the protein. *Aim.* In this study we set out to determine whether elevated 2-HG resulting from *IDH1* mutations is also present in acute leukaemia. *Methods.* R132 mutation screening was performed by PCR-sequencing in 66 AML cell-lines and 48 normal karyotype AMLs. Intracellular 2-HG concentration in primary AML cells was determined using a LC/MS-MS technique. *Results.* The R132 mutation was not detected in any of the 66 AML cell lines tested, but was present in 4/48 (8.5%) normal karyotype primary cases of AML. Three samples showed a R132H mutation with one sample containing an R132C alteration. Sufficient stored material was available from *IDH1* mutated (n=3) and wild-type (n= 25) primary AMLs to determine intracellular 2-HG. Markedly elevated 2-HG was found in all three of the mutated cases (531, 919 and 1765 ng/10^6 cells). In contrast, in 25 of 25 primary AMLs without the mutation intracellular 2-HG was <17.5 ng/10^6 cells. In $IDH1^{WT}$ AML cell lines, exposure to exogenous 2-HG at concentrations of 1-10 µg/mL resulted in intracellular concentrations of 380-670 ng/10^6 cells. Although the uptake of 2-HG was not linear, the resulting intracellular concentration was similar to that seen in $IDH1^{MUT}$ cells. *Conclusions.* Markedly elevated 2-HG levels are found in AML cells heterozygous for mutations in *IDH1* and likely lead to elevated 2-HG in cells of the neighbouring microenvironment. We are currently investigating the mechanisms by which elevated 2-HG may be linked to leukaemogenesis or outcome.

0050

THE FOUR AND A HALF LIM DOMAIN PROTEIN 2 (FHL2) INTERACTS WITH CALM AND IS HIGHLY EXPRESSED IN ACUTE ERYTHROLEUKEMIA

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Background. The CALM/AF10 fusion resulting from the translocation t(10;11)(p13;q14) is found in acute myeloid leukemia (AML), T-cell acute lymphoblastic leukemia (T-ALL) and malignant lymphoma. The CALM/AF10 fusion gene has been shown to cause biphenotypic leukemia in a murine bone marrow transplant model. The CALM (Clathrin Assembly Lymphoid Myeloid leukemia gene) protein is a clathrin assembly protein which plays a role in clathrin-mediated endocytosis and trans Golgi network trafficking. AF10 is a putative transcription factor likely involved in processes related to chromatin organization. *Aim/Method*. To learn more about the function of CALM/AF10 fusion protein, we searched for protein interaction partners of CALM using a yeast-two-hybrid screen. *Results.* The four and a half LIM domain protein FHL2 was identified as one of the putative CALM interacting partners. The CALM-FHL2 interaction was confirmed by GST pull-down and co-immunoprecipitation experiments. Additionally, in co-localization studies CALM and FHL2 could be shown to co-localize in the cytoplasm. Gene expression profiling (Affymetrix based) of chronic myeloid leukemia (CML) and AML samples showed high expression of FHL2 in CML and in AML samples with complex aberrant karyotypes compared to AML with normal karyotypes or balanced chromosomal translocations. The higher expression of FHL2 in CML and in AML with complex aberrant karyotypes did not reach statistical significance at the 0.05 level but suggested a trend, which was then examined in a larger patient cohort (n=308). This analysis showed a significantly higher FHL2 expression in patients with AML M6 (acute erythroid leukemia) and also in AML patients with complex aberrant karyotypes compared to AML patients with normal karyotypes. Erythroleukemia and AML with complex aberrant karyotypes have a poor prognosis. Interestingly, high FHL2 expression in breast- gastric-, colon-, lung- as well as in prostate cancer has also been shown to be associated with an adverse prognosis. *Conclusions.* The interaction of FHL2 and CALM/AF10 makes it tempting to speculate that this interaction is in part responsible for the poor prognosis of CALM/AF10 positive leukemias. FHL2 is highly expressed in poor prognosis leukemias like erythroleukemia and AML with complex aberrant karyotypes.

0051

GENOME WIDE STUDY OF DNA METHYLATION IN AML

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DNA methylation is the most stable epigenetic modification and has a major role in cancer initiation and progression. The two main aims for this research were, firstly, to use the genome wide analysis of DNA methylation as a method for better understanding the development of acute myeloid leukemia (AML). The second aim was to detect differentially methylated genes between the subtypes of AML and normal bone marrow. We used the methylated DNA immunoprecipitation technique followed by high-throughput sequencing by Illumina Genome Analyser II (MeDIP-seq) for 9 AML patients for whom ethical approval has been obtained. The samples include 3 processing t(8;21), 3 t(15;17) translocations and 3 normal karyotype (NK) plus 3 Normal Bone Marrow (NBMs) from healthy donors. The number of reads generated from Illumina ranged between 18- 20 million paired-end reads/lane with a good base quality from both ends (base quality >30 represent 75%-85% of reads). The reads have been aligned using 2 algorithms (Maq and Bowtie) and the methylation analysis was performed by Batman software (Bayesian Tool for Methylation Analysis). The preliminary results of 4 cases of leukemia [2 of t(8;21), one case of t(15;17), one case of NK] and a NBM sample showed that the average promoter methylation in the 5 samples ranged between 0.3-0.5 (Batman score >0.6 equals methylation). The median of chromosomes' methylation in the NK leukemia was significantly less than the other 4 cases (P<0.05). An inverse correlation was observed between CpG

observed/expected ratio and promoter methylation in both leukemic patients and in NBM (Spearman r= -0.6201, P<0.0001). The investigation of the 35,072 promoter regions identified 740 genes, which showed a significantly higher methylation level in leukemic cases in comparison to NBM (P<0.0001). Among these genes previously identified as having distinctive methylation patterns in cancer e.g. DLK1, PITX2, MYOD1, AMN, MAEL, TERT and CYP1B1. The full results of genome wide analysis of the 12 samples will be presented.

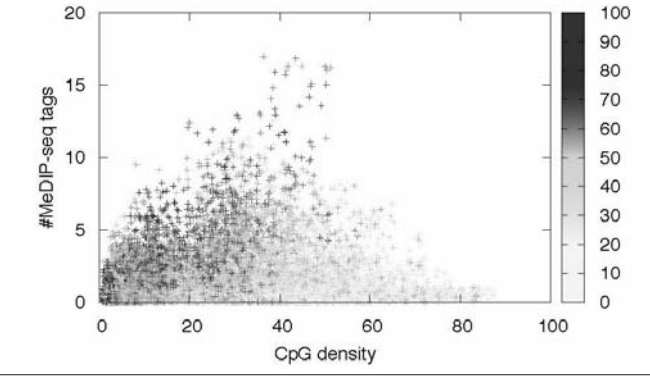

Figure. Batman calibration of MeDIP signals.

0052

NUCLEOPHOSMIN (NPM) IS UNIVERSALLY DEREGULATED IN ACUTE PROMYELOCYTIC LEUKEMIA

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Background. NPM is a multifunctional nucleolar phosphoprotein with roles in ribosome biogenesis, centrosome duplication, p53 response and DNA repair, and is commonly mutated in Acute myelogenous leukemia (AML). NPM is juxtaposed with retinoic acid receptor α (RARα)) in Acute Promyelocytic Leukemia (APL), raising the possibility that NPM functions are disrupted in this leukemia. *Aims.* We therefore sought to determine the extent of NPM deregulation in the U937-NPM-RARα+ cell line model, and to extend this analysis more generally to APL. Methods and *Results.* Immunofluorescent microscopy (IF) demonstrated abnormal distribution of NPM in patient-derived APL cells carrying NPM-RARα or PML-RARα, as well as cell lines expressing X-RARα: NPM was distributed into abnormally large aggregates within the nucleus and/or throughout the cytoplasm. NPM distribution reverted to normal after 48 hr of treatment with 1.0 μM all-trans retinoic acid (ATRA). NPM protein levels were also significantly increased in X-RARα+ cell lines. This is likely due to an alteration at the protein level, as NPM mRNA expression was unaffected by X-RARα, and no mutations were detected in the NPM locus. Indeed, we found that NPM protein half-life was increased in U937 cells expressing X-RARα. Taken together, these data suggested a potential disruption in nucleolar architecture in APL cells. Alterations in size and number of nucleolar organizing regions (NORs) in NB4 and U937-X-RARα cells were evident by light microscopy, when compared to U937 controls. Defects in nucleolar organization may lead to altered ribosome biogenesis, protein synthesis, as well as cell size and proliferation. Ribosomal RNA precursor expression was found to be increased significantly in U937-NPM-RARα cells, compared to controls. Relative cell volume, assessed by a flow cytometric assay, was moderately increased, by approximately 10% (mean of 5 independent replicates) in NPM-RARα$^+$ cells, while cell proliferation rates were significantly increased, and doubling time decreased, compared to U937 controls. Analysis of protein synthesis rates in U937-NPM-RARα$^+$ cells indicated that the incorporation of a fluorescent Methionine analogue was twice that in control U937 cells. Finally, in order to determine the applicability of our *in vitro* functional assays to APL patients, we analyzed pre-rRNA and 18S rRNA expression in bone marrow RNA extracted at diagnosis from 16 APL patients (10 BCR1/2 and 6 BCR3), in order to determine whether evidence of elevated nucleolar function were found in APL. When taken as a group, 10/16 APLs had elevated 18S rRNA levels (>1.5-fold compared to normal BM); 8/16 had similarly elevated pre-rRNA levels. Overall, pre-rRNA levels were elevated an average of 2-fold compared to normal controls, while 18S rRNA levels were elevated an average 1.8-fold (P<0.05 for both comparisons). *Summary/Conclusions.* We therefore present the first evidence that NPM may be universally deregulated in APL, leading to defective NPM function within the leukemic cell.

0053

IDENTIFYING MECHANISMS OF MLL-FUSION ONCOGENICITY IN ACUTE MYELOID LEUKAEMIA

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The MLL gene, located on 11q23, is involved in a large number of chromosomal translocations, including t(9;11)(p22;q23) and t(11;19)(p22;q23). These translocations encode the MLL-AF9 and MLL-ENL fusion transcription factors and are prevalent in infant acute leukaemia and treatment-related leukaemia. Leukaemias associated with these translocations have a particularly poor outcome. In order to identify novel mediators of MLL-fusion oncogenic activity, and gain a more complete understanding of the molecular pathways involved, we developed a system for conditional expression of the MLL-ENL fusion. Using a dual retroviral 'Tet-Off' system, we have previously immortalised primary mouse haematopoietic progenitor cells (HPC) and demonstrated that loss of MLL-ENL expression resulted in decreased HoxA gene expression, terminal myeloid differentiation *in vitro* and reversal of established leukaemia *in vivo*. These experiments have now been extended to generate conditionally immortalised MLL-AF9 myeloid cell lines. We aimed to define critical target genes involved in the immortalisation pathways of MLL-fusion oncogenes, by analysing both MLL-AF9 and MLL-ENL immortalised cells. Global gene expression analysis after treatment of immortalised cells with Doxycycline, confirmed the dependency of known transcriptional target genes, such as genes of the HoxA cluster, Meis1 and cMyb, on the presence of MLL-fusions. In addition our analysis revealed the differential expression of a number of genes known to be involved in cancer, for example Msi2. We have used shRNA knock-down of these target genes to analyse survival, proliferation and differentiation of the immortalised cells. This study will establish whether interfering with identified target genes may offer novel approaches to disrupt MLL-fusion activity and offer new targets for therapeutic strategies.

0054

REGULATION OF THE LEUKEMIA ASSOCIATED ETO NUCLEAR REPRESSOR GENE IS DRIVED BY GATA-1 IN ERYTHROID/ MEGAKARYOTIC CELLS

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Background. The Eight-Twenty-One (ETO), a nuclear co-repressor gene, is part of the AML1-ETO fusion protein produced by the chromosomal translocation t(8:21) in acute myeloid leukemia. ETO gene consists of 13 exons distributed over 87 kb of genomic DNA and belongs to the ETO homologue family. Expression of this gene has been reported in a variety of tissues as well as in erythroid cells. The normal function of ETO is essentially unknown and the gene regulatiom is also unknown. *Aims.* We tried to identify structural and functional promoter elements upstream of the coding sequence of the ETO gene in order to explore lineage-specific hematopoietic ETO expression to give clues to function. *Methods.* Transcription start site (TSS) was identified by RNA Ligase Mediated Rapid Amplification of c-DNA Ends (RLM-RACE). Reporter constructs of ETO proximal promoter upstream to the luciferase reporter gene in pGL3 Basic vector was made and activity was tested in hematopoietic cells. Chromatin immunoprecipitation (ChIP) and Electrophoretic mobility shift assays (EMSA) were performed to identify the promoter driving transcription factors. *Results.* A putative proximal ETO promoter was identified upstream of the transcription start site. Hematopoietic expression of an ETO promoter was specifically observed upon transfection in erythroid/megakaryocytic cells, which have appropriate endogeneous ETO gene activity. Results from electrophoretic mobility shift and antibody supershift assays showed GATA-1 of the nuclear extracts of erythroid/megakaryocytic cells to bind *in vitro* to probe including the GATA site within the conserved region of the ETO promoter. Furthermore, results from chromatin immunoprecipitation showed GATA-1 binding *in vivo* to elements within the conserved region of the ETO promoter. The results suggest that GATA-1 may have a role in activation of the ETO gene in cells

with erythroid/megakaryocytic potential. Leukemia associated AML1-ETO fusion gene strongly suppressed the ETO promoter in erythroid/megakaryocytic cells. *Conclusions.* We demonstrate that the GATA-1 transcription factor binds and transactivates the ETO proximal promoter in an erythroid/megakaryocytic-specific manner. Thus, trans-acting factors that are essential in erythroid/megakaryocytic differentiation govern ETO expression. We speculate that ETO is involved in repressing genes associated with self-renewal and proliferation. Suppression of the ETO gene by AML1-ETO could facilitate the AML1-ETO induced block of erythroid lineage commitment.

0055

THE IMPACT OF LENGTH AND INTEGRATION SITE OF THE INTERNAL TANDEM DUPLICATION OF FLT3 GENE ON THE CLINICAL OUTCOME OF PATIENTS WITH AML

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Background. FLT3/ITD is known to confer poor prognosis in AML patients owing to higher incidence of relapses, shorter disease free survival and overall survival (OS). However, there may be differences in outcome even within the *FLT3*/ITD$^+$ group of patients. *Aims.* We have analysed the impact of the length and integration site of ITDs on patients' clinical outcome. *Methods and patients*. The presence of *FLT3*/ITD was examined by RT-PCR. Positive patients were recognized according to abnormally longer PCR products in gel electrophoresis and confirmed by direct sequencing. We have studied 93 AML patients positively screened for *FLT3*/ITD. The median age at diagnosis was 51.5 (18.3-81.0) years; the initial median WBC count was 56.6×10^9/L ($0.7\text{-}488.0\times10^9$/L). 17/93 (18.3%) patients had APL. *Results.* The majority of *FLT3*/ITD$^+$ cases had a single duplication. Two ITDs were present in 11 (11.4%) cases and one patient carried three different ITDs. 5 patients lacked the wild-type allele. The size of ITDs varied between 12-120 bp. Borderline length of 39 bp was set up, and in patients with more than one ITD, the longest was used for further analyses. WBC counts at diagnosis were not influenced by the length of ITD. Among non-APL AML (AML) cases, patients with ITD ≤39 bp had lower complete remission (CR) rate (13/29; 44.8%) compared to those with longer ones (25/39; 64.1%; P=0.0567). In contrast, among APL patients, lower CR rate was observed in patients with longer ITDs (5/8; 62.5% vs. 7/8; 87.5%; P=0.1241). In AML, relapses insignificantly more frequently occurred in cases with longer ITD (13/26; 50.0%) than in those with shorter ones (5/13; 38.5%; P=0.2478). An opposite result was again found in APL patients, 3/7 cases with ITD ≤39 bp relapsed whereas no relapse among 5 patients with longer ITD was observed (P=0.0455). The length of ITD influenced OS neither in APL nor in AML subgroups. Among 81 patients with a single *FLT3*/ITD, 61 (75.3%) had their ITDs integrated in juxtamembrane (JM) domain (between codons 572-609) and 20 cases within the tyrosine kinase (TK) domain 1. These patients had significantly higher WBC counts at diagnosis (medians 107.7 vs. 54.3×10^9/L; P=0.0334). Patients carrying ITD in JM domain more easily reached CR (34/60; 56.7% vs. 8/20; 40.0%; P=0.0981). Relapses were observed in 6/9 (66.7%) patients with TK1 and in 12/34 (35.3%) cases with JM domain insertions (P=0.0449). The OS was longer in cases with ITDs integrated within JM domain (10.7 vs. 1.8 months; P=0.0774). *Conclusions.* We have confirmed inferior outcome of AML patients with ITDs integrated within the TK1 domain (codons 610-615). They more often relapsed and accordingly, had shorter OS than patients with ITDs in JM domain. The longer ITDs tended to correlate with higher CR rates and with more relapses, therefore the resulting OS was nearly identical in patients with longer and shorter ITDs. The opposite holds true for APL: less CRs and less relapses were seen in cases with longer ITDs. The position rather than the length of *FLT3*/ITD can prognostically stratify *FLT3*/ITD$^+$ AML.

0056

ABERRANT EXPRESSION OF THE CAUDAL-LIKE HOMEOBOX GENE CDX4 INDUCES ERYTHROID LEUKEMIA IN THE MURINE BONE MARROW TRANSPLANTATION MODEL

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Background. In normal hematopoiesis, balanced expression of developmental factors such as homeobox genes is important for fate decision. Aberrant expression of these factors has been shown to contribute to development of leukemia. The caudal-like Cdx members are important upstream regulators of Hox genes during embryogenesis and as such have the potential to act as potential oncogenes. *Aim.* In this study the effect of aberrant *Cdx4* expression on murine hematopoiesis was to be analyzed *in vitro* and *in vivo* using the murine BM transplantation model. *Methods.* To test the impact of aberrant Cdx4 expression on the level of murine progenitors, 5-FU enriched BM cells were retrovirally transduced with Cdx4 as well as empty vector control and analyzed by *in vitro* and *in vivo* assays. Expression of Cdx4 in different hematopoietic subpopulations was analyzed by qPCR and microarray experiments were performed to identify possible target genes. *Results.* Expression analyses showed high expression of Cdx4 in early murine hematopoietic progenitors followed by a highly significant downregulation towards the more differentiated hematopoietic stages (P=0.005). Among different lineage positive hematopoietic subpopulations, Cdx4 was lowest expressed in Ter119$^+$ erythroid precursors. *In vitro*, overexpression of *Cdx4* conferred proliferative potential to BM progenitors in liquid expansion assay. Expression of Cdx4 conferred serial replating capacity to murine BM progenitors compared to empty vector control (CFU total after 2nd replating: $2.6\times10^7\pm3.2\times10^6$ SEM/ 500 input cells in 1st CFC, n=8). Interestingly, immunophenotyping of the colonies revealed a significant 4.1-fold increase of erythroid Ter119$^+$ cells in 1° and 72.9-fold increase in 2° CFC (n=4, P=0.02 and 0.05, respectively). Even after 4th CFC colonies were positive for expression of Ter119. Lethally irradiated mice received 16±6% SEM Cdx4-GFP$^+$ BM cells together with GFP$^-$ helper cells. PB analysis after four week post transplantation revealed a GFP-positivity of 41.8±6% SEM (n=11) indicating a growth advantage of Cdx4-overexpressing BM cells. Furthermore, immunophenotypic analysis of GFP$^+$ cells showed multilineage engraftment indicating a role of Cdx4 in the expansion of short-term progenitors without perturbation of their hematopoietic differentiation program. In contrast to the data previously published by Bansal *et al*. in BALB/c mice where Cdx4 was shown to induce AML with myelomonocytic features, all our Cdx4-transplanted mice died with a median latency of 309 days which by Ter119 staining was histopathologically diagnosed as erythroid leukemia (n=10). Furthermore immunophenotyping revealed the majority of the leukemic cells to be positive for CD71 expression. Secondary mice died after a median of 74 days. At time of death, Cdx4-transplanted mice displayed splenomegaly with massive erythroid infiltration, a severely decreased lymphoid:myeloid ratio <1:5 and presence of erythroid blasts in PB, BM and spleen. Gene expression profiling of BM progenitor cells transduced with Cdx4 showed deregulation of genes involved in signal transduction processes as well as leukemogenic *Hox* genes compared to empty vector control. *Conclusions.* Overexpression of Cdx4 confers serial replating capacity to transduced murine BM progenitor cells and *in vivo* induces erythroid leukemia. This suggests Cdx4 to be a novel factor in the development of erythroid leukemia.

0057

THE CALM/AF10 INTERACTOR CATS IS A SUBSTRATE OF KIS, A POSITIVE REGULATOR OF CELL CYCLE PROGRESSION IN LEUKEMIA CELLS

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Background. CATS is a phosphoprotein initially identified in a Y2H screen as the CALM (PICALM) interacting protein expressed in thymus and spleen. The CATS interaction region of CALM is contained in the leukemogenic fusion protein CALM/AF10, which is found in acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL) and in malignant lymphoma. CATS sequesters CALM/AF10 in the nucleolus and interferes with the transactivation capacity of CALM/AF10 in a dose-dependent manner. However, the involvement of CATS in malignant transformation seems to go beyond its interaction with CALM/AF10. CATS is highly expressed in leukemia, lymphoma and tumor cell lines but not in non-proliferating T-cells or PBLs. The protein levels of CATS are cell cycle-dependent, induced by mitogens (e.g. PHA) and correlate with the proliferative state of the cell. Thus CATS can be viewed as a marker for proliferation. Aim. To further study CATS function, we searched for CATS interacting proteins. In addition, we analyzed KIS protein expression in primary cells from MDS and leukemia patients. *Methods and Patients.* CATS was used as a bait

in a yeast two-hybrid screen. Protein interaction identified was confirmed by coimmunoprecipitation of overexpressed proteins. *in vitro* kinase assay was performed to investigate whether CATS is a substrate of KIS and to map the residue within CATS, which is phosphorylated. KIS expression was analyzed on bone marrow mononuclear cells (MNCs) of MDS, AML and ALL patients by Western blotting. We studied 05 healthy donors, 11 MDS patients (07 low-risk [RA/RARS] and 04 high-risk [RAEB/RAEBt] according to FAB classification), 07 AML and 1 ALL. *Results.* We identified the kinase interacting with stathmin (KIS or UHMK1) as a CATS interacting partner. KIS is a nuclear serine/threonine kinase that possesses an RNA recognition motif and phosphorylates and regulates the activity of RNA associated factors. Moreover KIS positively regulates cell cycle progression through phosphorylation of p27KIP in leukemic cell lines. We confirmed the CATS-KIS interaction in the yeast system and by co-IP for both CATS isoforms. Using kinase assay we could show that CATS is a substrate for KIS being strongly phosphorylated on its serine 131, which lies within the SGSP consensus sequence for KIS phosphorylation. Finally, Western blotting analysis revealed elevated levels of KIS in MDS, AML and ALL compared to the control samples. *Summary/Conclusions.* Our results show that CATS not only interacts with but is also a substrate for KIS, suggesting that CATS function might be modulated through phosphorylation events. The identification of the CATS-KIS interaction further supports the hypothesis that CATS plays an important role in the control of cell proliferation. Moreover the elevated levels of KIS in hematological malignances suggest that KIS could regulate CATS activity and/or function in highly proliferating leukemic cells. Thus our results indicate that CATS function might be important to understand the malignant transformation mediated by CALM/AF10.

0058

I-B KINASE OVERCOMES PI3K/AKT AND ERK/MAPK TO CONTROL FOXO3A ACTIVITY IN ACUTE MYELOID LEUKEMIA

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Background. The human FOXO transcription factors, which include FOXO1, FOXO3a, FOXO4 and FOXO6, function as tumour suppressors by upregulating genes involved in cell cycle control (p27Kip1 and p21Cip1) or apoptosis (Fas-L and Bim). Loss of FOXO function due to genetic defects or posttranslational modifications are commonly observed in cancers. In acute myeloid leukemia (AML), chromosomal breakpoints involving FOXO3a and FOXO4 result in the suppression of FOXO transcriptional activity. Furthermore, deregulation of oncogenic kinases such Akt, ERK1/2 and IKK is frequently observed in AML. Interestingly, these kinases have been proposed to negatively regulate FOXO3a activity in most models by promoting its translocation from the nucleus to the cytoplasm and its proteasomal degradation via phosphorylation on different residues. *Aims.* We speculated that loss of FOXO3a tumour suppressor function may be a common feature of AML. We therefore analysed FOXO3a regulation by the PI3K/Akt, ERK/MAPK and IKK signaling pathways in both primary AML samples and a MV4-11/FOXO3a-GFP cell line. *Methods.* Bone marrow samples were obtained from 38 newly diagnosed AML patients included in trials initiated by the French GOELAMS group. PI3K/Akt ERK/MAPK and IKK signaling pathways were inhibited respectively using IC87114, UO126 and a specific IKKγ/NEMO-antagonistic peptide (referred as anti-Nemo). FOXO3a localization was tested using immunofluorescence. Primary AML cells and MV4-11 cells were infected with a lentivirus expressing either a FOXO3A-GFP or a FOXO3aS644A-GFP fusion protein. Expression of p21Cip1 and Fas-L mRNA was assessed on purified GFP^+ blast cells using qRT-PCR. Blast cell proliferation and apoptosis were quantified by [3H]-thymidine incorporation and annexin-V staining respectively. *Results.* We focused our study on FOXO3a as we found, using qRT-PCR and Western Blot analysis, that it was the only FOXO protein constantly expressed in primary AML cells. We observed in all AML samples tested that FOXO3a is inactivated due to its cytoplasmic localization. This inactivation of FOXO3a is not due to deregulation of the PI3K/Akt or ERK/MAPK signaling pathways as neither PI3K/Akt nor ERK1/2 specific inhibition results in FOXO3a nuclear translocation. In contrast, specific inhibition of IKK with the anti-Nemo peptide induces FOXO3a nuclear localization thereby suggesting that the deregulated activity of IKK in leukemic cells could be responsible for the inactivation of FOXO3a via its phosphorylation on S644. To confirm this result, leukemic cells from one patient and MV4-11 cells were infected with a lentivirus expressing a FOXO3aS644A-GFP mutant protein in which the IKK phosphorylation is abrogated. We found that the FOXO3aS644A-GFP protein is localized primarily within the nucleus in both AML cells and MV4-11 cells infected whereas FOXO3a-GFP protein is retained in the cytoplasm. This nuclear localization of FOXO3aS644A-GFP protein in MV4-11 cells correlates with an increased mRNA level of the FOXO3a target genes p21Cip1 (2.3-fold) and Fas-L (2.4-fold). Accordingly, FOXO3aS644A-GFP protein expression markedly decreased cell proliferation (reduction of 67%) and induced a slight but significant apoptotic response (2.5-fold increase; P=0.011). *Conclusion.*. Our results show that IKK controls FOXO3a activity and emphasize the control of FOXO3a activity as a new mechanism implicated in the pro-leukemic effects of IKK deregulation in AML.

0059

OVEREXPRESSION OF EVI-1 AND MN1 MAY PREDICT WORSE OUTCOME IN *DE NOVO* AML WITH MLL TRANSLOCATIONS

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Background and purpose Our previous study showed that overexpression of BAALC and MN1 predicted worse outcome in *de novo* AML with partial tandem duplication of MLL (Blood 114: 415a, 2009). The prognostic relevance of overexpression of FLT3, BAALC, EVI-1, MN1, FHIT and ERG genes in AML patients with MLL translocation (MLL-T) was not clear. We aimed (1) to measure the mRNA expression levels of these genes in AML patients with MLL-T, (2) to compare the expression levels of these genes with normal controls, and (3) to determine their prognostic significance. *Patients and Methods.* Bone marrow samples from 60 *de novo* AML patients with MLL-T at diagnosis were analyzed. The MLL fusion partners included AF4 (n=2), AF6 (n=10), AF9 (n=19), AF10 (n=8), ELL (n=10), ENL (n=3), and other rare subtypes (n=8). Real-time quantitative PCR assay with TaqMan probe was performed to measure the expression of genes in patients and controls. The expression levels of target genes were calculated as the copy number of each gene normalized to the copy number of ABL control gene and were dichotomized at the median value to low and high expression groups. The event-free survival (EFS) and overall survival (OS) were compared between the two groups. *Results.* MLL-T patients had significantly higher expression levels of FLT3 (P<0.0001), EVI-1 (P<0.0001) and a borderline higher level of MN1 as compared to controls (P=0.053) (Table 1). The EFS of the 48 patients who received standard induction chemotherapy was 33.0±8.7 mos (95% CI: 15.9 - 50.1 mos) and OS was 36.2±7.9 mos (95% CI: 20.7 - 51.7 mos). The CR rates were significantly different between low and high expression groups for EVI-1 (P=0.019), a borderline significance for FHIT (P=0.072) and MN1 (P=0.059), but no significant differences for FLT3, BAALC, or ERG. There were a borderline difference in EFS between high and low expression of EVI-1 (P=0.087) and MN1 (P=0.068). Patients with low expression of EVI-1 had a borderline longer OS than those with high expression levels (p = 0.066). No significant difference in OS was observed for MN1 expression. The EFS and OS were not significantly different between low and high expression groups for FLT3, BAALC, FHIT, and ERG. *Conclusions.* Our results showed that in MLL-T, there were overexpression of FLT3, and EVI-1, and a borderline overexpression of MN1. The patients with lower expression levels of EVI-1 and MN1 had a higher CR rate and favorable survival.

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Table 1. The expression levels of genes in MLL-T AML at diagnosis.

Gene	MLL-T (Mean±SE)	Control (Mean±SE)	*P*
FLT3	6.19±0.76 (N=59)	0.66±0.06 (N=43)	<0.0001
BAALC	0.42±0.12 (N=59)	0.27±0.04 (N=43)	0.257
EVI1	6286.14±1179.96 (N=46)	219.92±27.64 (N=35)	<0.0001
FHIT	0.25±0.05 (N=58)	0.28±0.03 (N=43)	0.688
MN1	0.10±0.02 (N=51)	0.06±0.01 (N=41)	0.053
ERG	1.43±0.24 (N=57)	1.63±0.20 (N=42)	0.553

0059a

FISH TO INVESTIGATE TET2 INVOLVEMENT IN THERAPY-RELATED MYELODYSPLASTIC SYNDROMES (T-MDS) AND ACUTE MYELOID LEUKEMIA (T-AML)

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Recently, deletions/mutations of the TET2 gene, a tumour suppressor gene mapped at band 4q24, have been revealed as an early event in the pathogenesis of many disparate myeloid disorders. TET2 mutations are discovered in 24% of t-MDS/t-AML patients whereas deletions in only 5% of patients. Based on these findings, the present study was aimed at establishing the incidence of band 4q24 deletions/structural defects in a series of 93 t-MDS/t-AML examined between January 1993 and January 2009 and to test whether TET2 deletions were correlated with any peculiar gene mutation and clinical findings. Our patients were forty-seven females and forty-six males, whose median age was 59 years (range 25-78). Nine patients had received radiotherapy (RT) only, fifty-one chemotherapy only and thirty-three both treatment modalities. Overall, alkylating agents (AA) were given to sixty-three patients, topoisomerase inhibitors (TI) to twenty-five and antracyclines (A) to five patients. Patients treated with AA developed t-MDS after a median time of 66 months (range 55-78) and t-MDS had a median duration of 11 months (range 4-17). In contrast, patients treated with TI and A developed t-AML without a preceding t-MDS after a median time of 18 months (range 12-26). At our observation, eighty-five patients presented with t-AML and eight patients with t-MDS. On clinical diagnosis, 81 patients (84.9%) presented clonal cytogenetic abnormalities involving chromosome 5 only (22.3%), chromosome 7 only (30.1%), both chromosomes (25.8%) and recurring balanced rearrangements (16.1%). A structural defect of chromosome 4 was revealed by conventional cytogenetics (CC) in three patients. A der(4)t(1;4)(p22;q23) and a t(3;4)(q21;q24) were revealed in one patient each, a 4q deletion in two. Up to now FISH with the 144B4 (mapped at 14q22.3), 810D13, 571L19, 414I7 (all mapped at 4q23), 356L5 and 16G16 (both covering the TET2 gene at band 4q24), 642P17, 788K3, 752J12 (all mapped at 4q24) and 66J16 (mapped at 4q25) probes was carried out in 24 patients. All these probes were obtained from BACPAC Resources Center at C.H.O.R.I. (Oakland, USA), labelled and applied as previously reported. The cut-off values for interphase FISH (i-FISH) were obtained from the analysis of 300 nuclei from ten normal samples and were fixed at 10%. The patient with the unbalanced t(1;4) translocation showed that 88% of interphase and mitotic cells had lost the 356L5, 16G16, 788K3 and 642P17 probes and had maintained the 752J12 and 66J6 probes. So, this patient presented a loss of the TET2 gene and of the 788K3 and 642P17 probes even if the breakpoint of the chromosomal translocation was localized at band 4q25. The other three patients presented a cryptic deletion of the 356L5, 16G16 and 788K3 probes. In conclusion, i) FISH is a good method for identifying cryptic TET2 deletions, as the chromosomal area containing this gene is often deleted independently of the chromosomal breakpoints; ii) TET2 deletions are rare events occurring at an incidence of 5.1%. The 16% frequency of our study is probably due to bias in sample collection; iii) TET2 deletion is not always associated with chromosome 4 rearrangements on conventional cytogenetics.

Acute myeloid leukemia - Clinical 1

0060

PROGNOSTIC VALUE OF THE ALLELIC BURDEN OF FLT3 INTERNAL TANDEM DUPLICATION IN PATIENTS WITH INTERMEDIATE-RISK CYTOGENETICS ACUTE MYELOID LEUKEMIA DEPENDS ON UNDERLYING MUTATIONAL STATUS OF NPM

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FLT3 internal tandem duplication (FLT3-ITD) confers an adverse prognosis to patients with intermediate-risk AML (IR-AML), with a high risk of relapse. Nonetheless, FLT3-ITD allelic burden has been reported to modulate the unfavourable prognosis of this mutation, and this effect might be influenced by NPM mutational status. In this context, we aimed to analyze the prognostic impact of the ratio FLT3-ITD/FLT3 wild type (FLT3wt) in IR-AML patients according to NPM status. We analyzed 409 patients (age: 51, 17-73) diagnosed with *de novo* IR-AML included in three consecutive CETLAM trials (LAM-94, LAM-99, and LAM-03), with available molecular information. NPM mutation (NPMmut), FLT3-ITD, CEBPA mutations (CEBPAmut), and MLL partial tandem duplication (MLL-PTD) were determined as previously described. The FLT3-ITD/FLT3wt ratio (FLT3 ratio) was defined as the ratio of the area under the curve of the mutated and wild-type peaks obtained by Genescan. NPMmut was detected in 205 patients (50%), 93 of them with concomitant FLT3-ITD (23%), 45 patients (11%) harbored a wild-type NPM (NPMwt) with FLT3-ITD, 25 patients had CEBPAmut, and MLL-PTD was identified in 11. In the overall series, complete response rate (CR), survival (OS), and relapse incidence (RI) were 82.6% (95% CI: 0.79-0.86), 40±4% (5-yr), and 48±5% (5-yr), respectively. Independent prognostic variables were age (P=0.006), WBC at diagnosis (<0.001), and NPM status (P=0.002) for CR achievement, and WBC at diagnosis (P<0.001, relative risk, RR=1.004, 1.002-1.007) and molecular category (NPMmut/FLT3wt vs. other; P<0.001, RR=2.79, 1.9-4.05) for relapse.

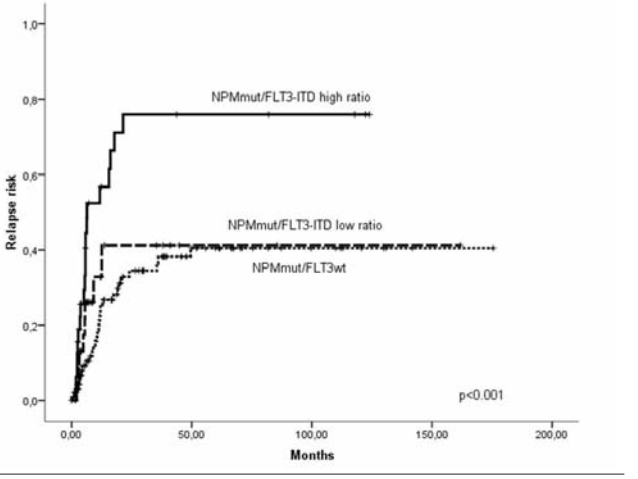

Figure.

Moreover, among patients with FLT3-ITD, FLT3 ratio showed independent prognostic value (P=0.037). The effect of FLT3 ratio on RI was further analyzed according to NPM status. For this purpose, FLT3 ratio was categorized in two groups (below or above the median value, 0.613). In patients with NPMmut, RI did not differ between patients with a low FLT3 ratio and those patients lacking FLT3-ITD (Figure). In accordance, two molecular categories were defined: a group of patients

with low-risk features (LOWRISK: NPMmut without FLT3-ITD or low FLT3 ratio, or CEBPAmut), and another group of high-risk molecular lesions (HIGHRISK: NPMwt regardless FLT3-ITD, high FLT3 ratio, or MLL-PTD). Thus, LOWRISK patients showed a better outcome compared to HIGHRISK patients, with an inferior RI (5-yr RI: 40±5% vs. 72±4%, P<0.001) and longer OS (5-yr: 58±4% vs. 31±4%, P<0.001). Finally, the effect of post-remission strategy in CR1 was analyzed according to this categorization. Thus, in LOWRISK patients, a similar outcome was observed after autologous stem-cell transplantation (HSCT) and allogeneic HSCT. On the contrary, in the HIGHRISK subgroup, alloHSCT in CR1 was followed by a significantly decreased RI (31±8% vs. 64±6%, P=0.001) and a trend to a longer survival (5-yr OS: 57±8% vs. 34±6%, P=0.07). In conclusion, FLT3-ITD allelic burden influences the outcome of patients with IR-AML, and this effect seems to interact with NPM mutational status. Thus, RI of patients with NPMmut IR-AML and low FLT3 ratio was similar to that of NPMmut IR-AML patients lacking FLT3-ITD. Therefore, estimation of FLT3 ratio might refine the prognosis determined by molecular markers, although its prognostic impact should be confirmed in a prospective fashion.

0061

DYNAMIC SINGLE CELL NETWORK PROFILES (SCNP) IN AML ARE ASSOCIATED WITH PATIENT RESPONSE TO STANDARD INDUCTION THERAPY

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Background. Complete response (CR) to induction chemotherapy is observed in approximately 60% of patients with FAB non-M3 Acute Myeloid Leukemia (AML) at diagnosis. However, no methods exist to predict with high sensitivity/specificity the disease response to standard AML induction chemotherapy at the level of individual patients. *Aims.* We performed a comprehensive functional assessment of intracellular signaling pathways to predict the likelihood of response to standard induction therapy in two sequential training cohorts of AML samples (N=34 and 88) *Methods.* Single Cell Network Profiling (SCNP) is an approach for analyzing and interpreting post-translational protein modifications (e.g. phosphorylation, acetylation etc.) at the single cell level. This technology allows simultaneous characterization of the range of critical cellular processes within AML, such as growth (cell cycle), viability (apoptosis), DNA damage, presence and function of drug transporters, and in-vitro effects of therapies on signaling networks. Using viable cells, measurements are made on endogenous proteins before and after exposure to extracellular modulators such as growth factors, cytokines or drugs. The modulators are meant to mimic the stimuli that the cell encounters in the body and are chosen to evoke a response from the cell that echoes how the signaling system is normally, or abnormally, patterned. The proteomic readout in the presence or absence of a specific modulator is termed "signaling node". Signaling nodes are evaluated within cells from samples that have associated relevant clinical information regarding response to the therapy of interest. Multivariate analysis can then be performed to create predictive models that can be validated in subsequent independent studies. *Results.* In the first study, univariate analysis identified multiple "signaling nodes"that correlated with response to induction chemotherapy (i.e. AUCROC ≥0.66; P≤0.05) at a level greater than age, a known factor associated with response to induction therapy. After accounting for age, similar findings were observed in the second study. For patients < 60 years old, CR was associated with the presence of intact apoptotic pathways. In patients ≥60 years old, non-response (NR) was associated with FLT3 ligand mediated increase in phospho (p)-Akt and p-Erk. Results were independent of cytogenetics, secondary AML, or FLT3 mutational status. Finally, we observed the value of multivariate models using independent nodes since they can provide improved sensitivity/specificity over single nodes performance. *Summary/Conclusions.* The data emphasize the value of quantitatively measuring single cell networks (SCN) under modulated conditions as a basis for the development of highly predictive tests for response to induction chemotherapy. These SCN profiles are predictive of disease outcome and distinct from other known prognostic factors such as age, secondary AML, and cytogenetics and importantly should be validated in future, independent studies.

0062

PHASE 1 STUDY OF SORAFENIB IN PATIENTS WITH REFRACTORY ACUTE LEUKEMIAS AND MYELODYSPLASTIC SYNDROMES

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Background. Activation of Ras/Raf/MEK/Erk (MAPK) signaling cascade is ubiquitous in cancer and represents attractive targets for cancer therapy. Inhibition of MAPK pathway has been shown to impair cell growth and survival of acute myeloid leukemia (AML) cell lines and primary samples with constitutive MAPK activation. Sorafenib is a Raf kinase inhibitor with additional inhibitory activity against fms-like tyrosine kinase 3 (FLT3) with internal tandem duplication (ITD) mutation. *Aim.* Define the maximal tolerated dose (MTD) for sorafenib in patients (pts) with relapsed/ or refractory acute leukemias and advanced myelodysplastic syndrome (MDS). *Methods.* Pts received one of two different schedules: Schedule "A": Once or twice daily, 5 days per week, every week; and Schedule "B": Once or twice daily, for 14 days every 21 days. Evaluation of response was done according to the modified International Working Group (IWG) criteria. Dose escalations were carried out in standard "3+3"design with a starting dose of 200 mg twice daily. Correlative studies using whole-blood samples were collected day 1, day 4 and day 14 during administration of the first course of sorafenib. Pts signed an informed consent. *Results.* From 2006 to 2009, 50 pts were enrolled (including 48 pts with AML) at UTMDACC in a phase I study: 31 to schedule A and 19 to schedule B. Median age was 61 years (range, 21 to 88 years) with a median 3 prior therapies. Forty pts had positive FLT3 ITD/ and or TKD mutation. Dose limiting toxicities were grade 3/4 hypertension, hyperbilirubinemia, and amylase elevation. The recommended phase 2 dose in hematologic malignancies is 400 mg twice daily for both schedules. Complete remissions (CR) or CR with incomplete recovery of platelets (CRp) were achieved in 5 pts. Significant reduction in bone marrow and/or peripheral blood blasts was seen in an additional 17 pts. Eleven of these responses (including 3 CR/CRp) lasted for 2 cycles or beyond. Two thirds of patients with AML and FLT3 ITD responded. Apoptosis induction and changes in mitochondrial membrane potential were significantly increased in peripheral blood mononuclear cells in patients with FLT3 ITD mutation on days +1 and +4 compared to baseline. *Conclusions.* Sorafenib is an active and well tolerated agent in AML with FLT3 ITD mutation. Sorafenib-based combinations are warranted in pts with AML, particularly in those with FLT3 ITD.

0063

IN ACUTE MYELOID LEUKEMIA, THE USE IN INDUCTION OF STANDARD DOSE CYTARABINE IS ASSOCIATED WITH A BETTER QUALITY OF RESPONSE AS COMPARED TO AN INDUCTION REGIMEN CONTAINING HIGH DOSE CYTARABINE

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Background. The clinical advantage of high-dose cytarabine (HDAC) in induction chemotherapy for acute myeloid leukemia (AML) is still controversial. *Aims.* The purpose of our study was to explore the impact on the "quality of response"of an induction regimen containing standard dose cytarabine (SDAC) versus HDAC by measuring minimal residual disease (MRD) once CR was achieved. *Methods.* MRD was determined by multiparametric flow cytometry on bone marrow samples collected at the end of induction and consolidation therapy. The threshold for MRD negativity was set below a number of 3.5×10^{-4} residual leukemic cells. We evaluated 123 patients with *de novo* AML, enrolled sequentially in DCE arm of AML10 (n=40) and in AML12 (n=83) EORTC/GIMEMA randomized trials between 1995 and 2007. In DCE arm of AML10, induction treatment combined AC (100 mg/m^2, day 1-10), etoposide (50 mg/m^2, day 1-5), and on days 1,3,5, daunorubicin (50 mg/m^2). In AML12 trial, patients received the same treatment of DCE arm except for AC dose that was 100 mg/m^2, day 1-10 or 3000 mg/m^2/q12 hrs on days 1, 3, 5, and 7 according to randomization. As consolidation, all patients received AC (500 mg/m^2/q12 hrs day 1-6) and daunorubicin (50 mg/m^2, day 4-6). Median age was 45 yrs (range 18-60), 73 males and 50 females. Seventy-five patients were treated with SDAC regimen and 48 with HDAC regimen. The two groups

were well balanced in terms of FAB distribution, WBC count, cytogenetics, FLT3 and NPM1 mutation and post-remissional transplantation therapy (autologous or allogeneic). *Results.* After induction, we observed a significantly (P=0.01) higher frequency of MRD negativity in SDAC arm vs HDAC arm (78.4% vs 21.6%, respectively). After consolidation, this figure was confirmed (78.6% vs 21.4%, P=0.005). At this stage, 4 further patients treated in the SDAC arm, became MRD negative, whereas only one of the HDAC arm did so. Overall, median level of MRD was significantly lower in SDAC group both after induction (1.1×10^{-2} vs 4.1×10^{-2}, P=0.02) and consolidation (5.3x10-3 vs 3x10-2, P=0.007). Based on the combination of MRD status after consolidation and AC schedule delivered, we identified 4 different groups of patients. Five years OS for SDAC-MRDneg, HDAC-MRDneg, HDAC-MRDpos and SDAC-MRDpos was 66%, 45%, 29% and 24%, respectively (P=0.008). Similarly, 5 years RFS for SDAC-MRDneg, HDAC-MRDneg, HDAC-MRDpos and SDAC-MRDpos was 66%, 45%, 34% and 18%, respectively (P=0.0002). Since 16 patients, 7 in the SDAC and 9 in the HDAC group, died because of toxic complications, cumulative incidence of relapse (CIR) was also evaluated. Five years CIR for SDAC-MRDneg, HDAC-MRDneg, HDAC-MRDpos and SDAC-MRDpos was 21% (95% CI, 19-21), 15% (95% CI, 12-24), 51% (95% CI, 55-60), and 75% (95% CI, 74-76), respectively (P<0.0001). *Conclusions.* Delivery of an induction regimen containing daunorubicin, etoposide and SDAC given for 10 days, results in a more efficient clearance of leukemic burden compared to a similar regimen HDAC based. Such superior efficiency translates into a better "quality"of response, as demonstrated by the more frequent achievement of a MRD negative status, and then into a more favorable outcome.

0064

PRIMING WITH ARSENIC TRIOXIDE IMPROVES THE OUTCOME OF NEWLY DIAGNOSED ACUTE MYELOID LEUKEMIA PATIENTS <60 YEARS OLD TREATED WITH HIGH-DOSE CYTARABINE AND IDARUBICIN

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Background. Constitutive signal transducer and activator of transcription (STAT) 3 activity was shown to be present in leukemia cells from approximately 50% of acute myeloid leukemia (AML) patients (pts) and to correlate with adverse treatment outcome. We have shown that arsenic trioxide (ATO) down-regulates STAT3 activity in AML cells within six hours with a reduction of cell survival manifested after 48 hours *in vitro*. We therefore hypothesized that *in vivo* exposure of AML cells to ATO prior to induction chemotherapy might sensitize the blasts to the chemotherapy agents. *Aim.* To conduct a phase I clinical trial to evaluate the biologically effective and/or the maximally tolerated dose of ATO administered prior to induction chemotherapy with high-dose cytarabine (Hidac) and idarubicin (Ida) for previously untreated AML in pts <60 years old. *Methods.* We compared retrospectively the outcome of the 61 <60 year old AML pts enrolled on our phase I clinical trial with ATO followed by Hidac/Ida to that of 118<60 year-old previously untreated AML pts undergoing induction therapy at Roswell Park Cancer Institute with Hidac/Ida without ATO. All pts signed informed consent. *Results.* There was no significant difference in the median age (47.0 vs. 45.5; Wilcoxon P=0.7) or presenting white blood cell count (9.0 vs. 17.8; Wilcoxon P=0.8) between pts treated with Hidac/Ida and those treated with ATO/Hidac/Ida. Similarly, the two cohorts had similar representation of pts with *de novo* AML (79% vs. 87%; Fisher P=0.2). Finally, the two cohorts had similar karyotype subgroup representation [favorable/intermediate/unfavorable 14%/48%/39% vs. 14%/51%/ 35%; Fisher P=0.9]. The complete remission (CR) rate was similar between the two cohorts (72% vs. 66%; Fisher P=0.40). A total of 41% of the Hidac/Ida pts proceeded to an allogeneic transplantation in first CR compared to 49% of the ATO/Hidac/Ida pts (Fisher P=0.3). The median follow-up for pts treated with Hidac/Ida was 17.4 months compared to 19.4 months for the pts treated with ATO/Hidac/ida (Wilcoxon P=0.7). Interestingly, the overall survival of pts treated with ATO/Hidac/Ida was significantly better compared to those treated with Hidac/Ida (median survival 39.9 vs. 17.6 months.; logrank P=0.039). *Summary.* Priming with ATO improved the outcome of <60 year old AML pts treated with Hidac/Ida compared to historical controls. These data, if reproduced in a randomized phase III clinical trial, suggest that priming the leukemia-initiating cells with ATO may be a means to enhance the effect of chemotherapy.

0065

TWO-STEP RESPONSE-ORIENTED INDUCTION PREDICTS LONG-TERM OUTCOME OF ADULT PATIENTS WITH STANDARD- AND HIGH-RISK ACUTE MYELOID LEUKAEMIA (AML): A NORTHERN ITALY LEUKAEMIA GROUP (NILG) STUDY

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Background. In AML it is unclear whether long-term survival of different risk groups is affected by complete remission (CR) being achieved after the first or second chemotherapy course, the approach to patients who fail a first conventional cycle is not standardized, and relatively few of these cases enter CR repeating the same induction programme. *Aims.* A two-step response-oriented induction was designed to optimize CR results, adopting a sequential high-dose schedule for patients refractory to standard chemotherapy cycle 1. This report describes the relationship between achievement of CR after cycle 1 or 2 and the probability of survival at 5 years in different risk groups. *Methods*. CR induction therapy consisted of (1) standard ICE therapy followed, in refractory cases, by (2) sequential HAI (ICE: idarubicin 12 mg/m^2/d dd 1-3, etoposide 100 mg/m^2/d dd 1-5, cytarabine 100 mg/m^2/bd dd 1-7, G-CSF from d 8; s-HAI: idarubicin 17.5 mg/m^2/d dd 1 and 8, cytarabine 3 g/m^2/bd dd 2, 3, 9, and 10, G-CSF from d 11). Patients were stratified as standard- or high-risk (SR, HR) according to cytogenetics (i.e. favourable, unfavourable) and additional risk factors in case of intermediate/normal/unknown cytogenetics (HR with any: WBC count >50×10^9/L, FAB class M0/6/7, hepato/splenomegaly, MDS-related/secondary AML, FLT3/ITD mutation; SR with none).

Survival probabilities at 5 years according to risk class (SR, HR) and CR achieved after cycle 1 or 2 (C1, C2) NR denotes refractory AML; all patients (A); patients with de novo AML aged <60 years (B)

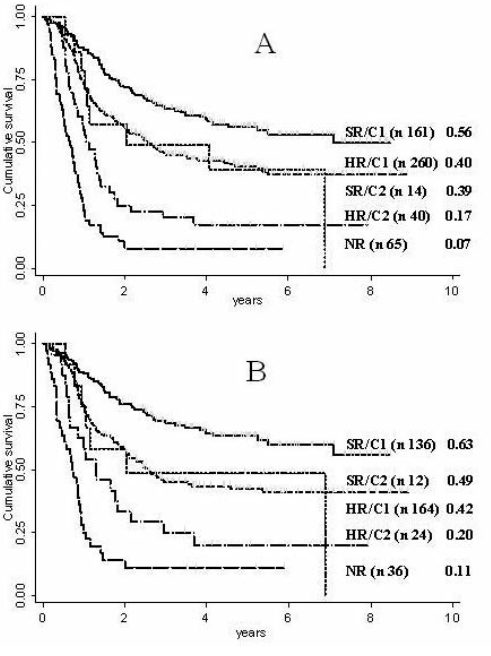

Figure 1.

Results. The prospective NILG-AML 01/00 study enrolled 581

patients, with median age 52 years (range 19-68). Cytogenetic risk groups were favourable 8.5% (n=50), intermediate/normal 59% (n=342; [SR=123; HR=219]), unfavourable 20.5% (n=120), unknown 12% (n=69 [SR=24; HR=45]). SR (n=197) and HR (n=384) groups differed significantly (P<0.05) with regard to WBC count (median 9.2 vs. 15.3), adverse FAB classification (0% vs. 15.5%), MDS/secondary AML (1% vs. 22%), hepato/ splenomegaly (5.5% vs. 28%), and FLT3/ITD$^+$ status (2% vs. 33%). After ICE cycle 1, CR was 82% in SR and 68% in HR (P=0.002) and 129 patients had resistant AML (22%); 95 ICE-refractory patients (73.6%; SR=22, HR=73) had s-HAI cycle 2, with a response rate of 64% in SR vs. 55% in HR (P=0.46). Thus final CR rate was 89% in SR and 78% in HR. Planned postremission therapy was allogeneic stem cell transplantation in HR and/or late responders, high-dose cytarabine cycles in SR early responders. Looking at long-term outcome, 5-year survival varied significantly according to both risk class and achievement of CR after cycle 1 or 2: 56% vs. 39% in SR (P=0.004), and 40% vs. 17% in HR (P=0.0000). The best outcome was observed in SR early responders aged <60 years with *de novo* AML (Figure 1). *Conclusion*s. Because the number of cycles to CR impacts on long-term survival of both SR and HR patients, the achievement of CR at cycle 1 should be a primary therapeutic endpoint of clinical studies. Patients who fail standard induction should not repeat the same regimen; for these cases, schedules like s-HAI yielding a >50% success rate are preferable.

0066

A PHASE II STUDY OF GTI-2040, AN ANTISENSE TO RIBONUCLEOTIDE REDUCTASE IN COMBINATION WITH HIGH-DOSE CYTARABINE IN RELAPSED OR REFRACTORY ACUTE MYELOID LEUKEMIA

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Background. GTI-2040 is a 20-mer antisense to the R2 component of ribonucleotide reductase (RNR) mRNA. RNR is required for the conversion of ribonucleotides to deoxyribonucleotides, a crucial step during DNA synthesis and repair. Cytarabine (AraC) is a cytotoxic agent converted into AraC triphosphate (AraCTP) and is the backbone of several regimens in acute myeloid leukemia (AML). AraCTP competes with deoxycytidine for DNA incorporation, and AraC cytotoxicity correlates with AraCTP incorporation into DNA. Therefore, we hypothesized that RNR downregulation by GTI-2040 results in decreased levels of endogenous deoxycytidine, leading to a preferential DNA incorporation of AraCTP and increased cytotoxic activity. *Aims.* To test this hypothesis, we undertook a multi-center, Simon two stage Phase II (P2) study of GTI-2040 plus high dose cytarabine (HiDAC) in patients with relapsed or refractory AML (ages 18-59 years). The primary objective was to determine the overall response rate (complete remission (CR) and CR with incomplete blood count recovery (CRi)) of GTI-2040 with HiDAC. Secondary objectives included pharmacokinetic (PK) studies including intracellular GTI-2040 and exploration of the combination's pharmacodynamic (PD) activity, including changes in dNTPs, NTPs, and AraCTP. *Methods*. Patients received HiDAC at 3 gm/m^2 every 12 hours for a total of 8 doses and GTI-2040 5 mg/kg/day continuous infusion (CI) for 6 days beginning 24 hours before HiDAC (P2 group). To further explore the PD activity of the combination, an additional PD group received the same HiDAC dose and schedule with GTI-2040 5 mg/kg/day CI for 4 days beginning 24 hours after HiDAC initiation. In the Stage 1 P2 arm, if 3 or more patients achieved CR/CRi, the regimen would be deemed worthy of further study. All patients provided informed consent. *Results.* Twenty-five patients were enrolled; 15 in the P2 group and 10 in the PD arm. Median age was 43 years (range 18-59 years); men comprised 72% of patients. Fourteen patients (56%) had primary refractory AML, 9 (36%) had relapsed less than 1 year after achieving first complete remission (CR1), and 2 (8%) had relapsed >1 year after achieving CR1. Overall, 15 (60%) pts had received prior HiDAC. Fourteen patients (56%) had intermediate risk cytogenetics, and 11 (44%) had adverse risk karyotypes by CALGB classification. Toxicities were comparable to those observed with HiDAC therapy alone. Grade 3/4 treatment emergent non-hematologic toxicities at least possibly related and seen in 2 or more patients included febrile neutropenia, catheter related infections, and reversible elevations in ALT/AST. Only 1 patient had a grade 3 reversible cerebellar toxicity. Overall, 7 (28%) patients (P2 arm n=4; PD group n=3) achieved CR/CRi (CR n=6; CRi n=1) and 1 patient achieved partial remission. Pharmacokinetic parameters and intracellular GTI-2040 concentration evaluated using an ELISA-based assay developed by our group, and dNTP/NTP, and AraCTP levels using a LC-MS/MS assay will be presented. *Summary/Conclusions.* The combination of GTI-2040/HiDAC was tolerable and met efficacy criteria for Stage 2 enrollment. Safety and efficacy demonstrated in this Phase II and prior Phase I in similar high risk AML patients supports further development in a larger randomized trial. [NCI R21 CA133879-01]

0067

MYELOID LEUKEMIA OF DOWN SYNDROME - MONITORING OF MINIMAL RESIDUAL DISEASE

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Background. Children with Down syndrome (DS) have a 5 to 10% risk of transient leukemia (TL) and a ~2% risk of myeloid leukemia (ML-DS). Children with ML-DS had a favorable outcome with less intensive chemotherapy compared to non-DS AMKL. Somatic mutations of the GATA1 gene were detected in almost all DS TL and ML-DS children's. *Aims.* To demonstrate the feasibility to monitor the GATA1 positive leukemic clone (minimal residual disease, MRD). We compared the MRD monitoring by qPCR, immunophenotyping and morphology. *Patients.* In Germany 56 children with ML-DS (f=26; m=34; age 1.56 years (0.7-4.0) were enrolled to the ML-DS 2006 registry. Treatment consisted of 4 courses of chemotherapy with significantly lower cumulative dosages of cytarabine (27.4 g/m^2) and anthracyclines (190 mg/m^2) compared to conventional protocols (AML-BFM 2004: cytarabin 47.3 g/m^2; anthracyclines 450 mg/m^2). The median WBC was 4.7g/l (range 1-160 g/L), Hb 9.5g/dL (3.1-13.4), platelets 45 g/L (1.5-257 g/L). Four children of the cohort had trisomy 21 mosaicism. *Methods.* Morphology, immunophenotyping and molecular genetics were centrally performed. For screening of GATA1 mutation a direct sequencing of exon 1, 2, and 3 was performed. If no mutation could e detected blasts sorting or subcloning was added. For monitoring of GATA1 mutant clone we used a qPCR with patient specific TaqMan probes and primers. The standard curve was a serial dilution series of initial patient DNA. We achieved a sensitivity of 10 -5 and quantitative range of 10 -4. As negative control we used the cell line 293T (Human embryonic kidney cell line) and healthy donor DNA. *Results.* GATA 1 mutations were confirmed in 54 patients (96%). GATA1 detection was not possible in 2 patients with initial blasts count less than 2% and lack of sufficient material. The 3-years-eventfree and overall survival were 94±3% and 96±2%, respectively. Two children died early due to infections. All others were in complete remission (follow-up 2 years; 0,2-4 yrs). Sixteen children had a history of TL (28%). In 4 out of them, the GATA1 clone have been successfully monitored by qPCR. All patients showed spontaneous reduction of blasts after TL, however never became negative (all time points >10^{-4}) by qPCR monitoring and developed ML-DS within 0.7 to 3.2 years. To date 15/56 children with ML-DS the GATA1 mutant clone has been monitored prospectively. By morphology and immunophenotyping the blasts decreased from 30.5% (5-76% at diagnosis to 2.4% (0.4-4.5%). After the 3rd element only one child remain MRD- positive. The qPCR confirmed the results, although the sensitivity was about 1-log higher). All 54 patients are still in continuous complete remission with a median follow-up of 2.3 years (0.5 to 4.3 years). *Conclusions*. This preliminary data demonstrates the feasibility to monitor MRD in children with ML-DS. The GATA1 clone specific qPCR showed a high sensitive and might help to define new risk groups in ML-DS aiming further reduction of treatment intensity.

0068

PHASE I CLINICAL TRIAL OF THE ANTI-CD33 IMMUNOTOXIN HUM195/RGEL IN PATIENTS (PTS) WITH ADVANCED MYELOID MALIGNANCIES

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Background. HuM195 is a humanized version of the mouse M195 anti CD-33 monoclonal antibody. CD33 is heavily expressed on the surface of myeloid leukemia cells. The anti-CD33 immunotoxin HuM195/rGel combines HuM195 with recombinant gelonin, a RNA glycosidase that

inhibits protein synthesis. *Aim.* The primary objective of this study was to determine the safety and toxicity of HuM195/rGel in pts with refractory or relapsed leukemia. *Methods.* Pts age ≥18 years with relapsed or refractory AML, RAEB-t, RAEB, CMML or CML-AP/BP and CD 33 expression ≥80% by flow cytometry, were eligible. Pts received doses of 2.5 to 10 mg/m^2 using a standard 3 + 3 design. Therapy was administered over 2 one-hour infusions per week for 2 weeks followed by a 2 week period of observation. Pts taken off study before 28 days for reasons other than toxicity were replaced for MTD calculations. *Results.* 28 patients were enrolled: 23 AML, 4 MDS, and 1 CMML. Median age was 68 years, median number of prior therapies was 2, median peripheral blood blasts 24%, median bone marrow blasts 39%, and median percentage of CD 33 was 95%. The highest achieved blood levels of HuM195/rGel were 200-300 ng/mL which cleared with a half-life of ~20 hrs. Two (<10%) out of 23 patients developed antibodies to the rGel portion of the drug as assessed by ELISA. Twenty two pts were evaluable for dose limiting toxicity (DLT). Two patients developed DLT (allergic reaction) at a dose of 10 mg/m^2, thus 7mg/m^2 was declared the maximum tolerated dose. Drug related adverse events at ≤7mg/m^2 include: grade ≤ 2 fever and chills at all dose levels. Six (27%) out of the evaluable patients had a ≥50% decrease in PB blasts or BM blasts. In addition, 4 pts had an increase in platelet count to >100×10^9/L and 4 pts demonstrated increased neutrophils to >1×10^9/L. There were no complete or partial responses. *Conclusions.* HuM195/rGel can be administered safely at doses up to 7 mg/m^2 with an improvement in the tumor load in some patients.

0069

FAVORABLE OUTCOME IN PATIENTS WITH ACUTE MYELOID LEUKEMIA WITH NPM1 MUTATION AUTOGRAFTED AFTER CONDITIONING WITH HIGH DOSE CONTINUOUS INFUSION IDARUBICIN AND BUSULPHAN

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Background. Mutations of the nucleophosmin gene (NPM1), in the absence of concurrent FLT3-internal tandem duplication (FLT3-ITD) have impressive prognostic value in patients with acute myeloid leukemia (AML), carrying normal karyotype (NK). However, while there is a general consent that allogeneic stem cell transplantation (SCT) has no role in patients with NPM1$^+$FLT3$^-$ AML in first complete remission (CR), no studies have specifically focused on the therapeutic results of autologous SCT (ASCT) in NK AML patients presenting with NPM1$^+$/FLT3$^-$ genotype. *Aims.* To describe treatment results from a series of 19 patients with NPM$^+$/FLT3$^-$ autografted in first CR after conditioning with a regimen, named BuI, based on high dose continuous infusion idarubicin and Busulphan and perform a comparison with a control group of 80 NK AML patients with no or different molecular abnormalities. *Methods.* Ninety-nine consecutive patients with NK AML, treated at our Institutions between April 2003 and June 2009, in which ASCT was actually given in first CR were analyzed. There were 62 male patients (64%) and 37 female patients (36%) with a median age of 54 years (range 15-77). Overall 83 patients (84%) were diagnosed as having *de novo* AML, while in 16 patients (16%) AML arose after a previously diagnosed myelodysplastic syndrome (MDS). *Results.* Overall, 19 out of 99 patients autografted after conditioning with BuI regimen (19%) had NPM1 mutation in absence of FLT3 mutations. The control group, accounting for 80 patients, included sixteen cases (15%) with both mutations, 10 (12%) with FLT3/ITD mutation and no NPM1 mutation, and 54 (68%) in whom neither NPM1 nor FLT3 mutations were detectable. Median time from CR achievement to ASCT was similar between the two groups (3 months, range 2-5 for NPM1$^+$ patients) as opposed to 3 for the control group (range 2-6), p: 0.87. The median survival (OS) for the whole patient population was 34 months, the median disease free survival (DFS) was 22 months. Median OS and DFS were significantly longer for patients with isolated NPM1 mutation as opposed to controls (OS: not reached vs. 25 months, P:0.02; DFS: not reached vs. 16 months, P:0.007, respectively). Of interest, patients with isolated NPM1 mutation had a better outcome in terms of either survival or DFS as compared to the group of 16 NMP1$^+$/FLT3$^+$ patients. Relapse post-ASCT occurred in 3 out of 19 patients with NPM1 isolated mutation (16%), 2 with *de novo* AML and 1 with AML post-MDS, as opposed to 49 of 80 in the control group (61%), P:0.001. Overall, second CR was achieved in 12 out of 52 relapsed patients (23%) and was almost exclusively limited to those whose first CR lasted more than 6 months. *Summary and Conclusions.* Our study suggest that BuI regimen results in favorable clinical outcome in patients with isolated NPM1 mutation and could be investigated in a randomized study versus other conditioning regimes or repeated courses of chemotherapy with high dose cytosine-arabinoside.

0070

VORELOXIN SINGLE-AGENT TREATMENT OF OLDER PATIENTS (AT LEAST 60 YEARS) WITH PREVIOUSLY UNTREATED ACUTE MYELOID LEUKEMIA: FINAL RESULTS FROM A PHASE 2 STUDY WITH 3 SCHEDULES

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Background. Voreloxin, an anticancer quinolone derivative that inhibits topoisomerase II, has demonstrated clinical activity in ovarian cancer and acute myeloid leukemia (AML). Final results are reported from this phase 2 study (N=113). *Aims.* Assess clinical activity and safety/tolerability of voreloxin treatment in 3 dosing schedules. *Methods.* Eligible patients provided informed consent and had AML (WHO subtype, except APL) considered unlikely to benefit from standard induction therapy, with ≥1 adverse risk factor: age ≥70, secondary AML, intermediate (I) or unfavorable (U) cytogenetics, or PS=2. Voreloxin was administered by short (≤10 min) IV infusion in 3 dosing schedules: A, weekly (qwk) ×3 (days [d] 1, 8, 15) at 72 mg/m^2 (N=29); B, qwk×2 (d 1, 8) at 72 mg/m^2 (N=35); and C, twice weekly (biw) ×1 (d 1, 4) at 72 (N=29; C72) or 90 mg/m^2 (N=20; C90) for up to 4 cycles. *Results.* Median age was 74 years, most patients were male with an ECOG PS 0 or 1, and cytogenetics were I (46%) or U (42%) per 2010 NCCN guidelines. The overall remission rate (ORR=CR+CRp [complete remission + CR with incomplete platelet recovery]) across all doses and schedules was 34% (range, 25-41%) and 74% of CR were in first cycle. Reinduction resulted in CR or CRp for 35% of those reinduced. By schedule, ORR was 41% for A, 29% for B, 38% for C72, and 25% for C90. No dose response was observed between C72 and C90. All-cause mortality rates were lowest in C72 (7% at 30 days, 17% at 60 days) compared with A, B, and C90. Median duration of remission (DR) was 10.7 months (mo) for A (median too early to evaluate [TETE] for B, C72, or C90). Median overall survival (OS) was 8.7 mo (A), 5.8 mo (B), 7.3 mo (C72, preliminary); C90 is TETE. One-year OS was 38% for A and TETE for other schedules. Across all schedules, the most common nonhematologic adverse events grade ≥3 were febrile neutropenia (49%) and infections: pneumonia (28%) and sepsis/bacteremia (28%). Overall safety profile was more acceptable for C72 than for A and appeared further improved over B. ORR was 30% for patients ≥75 years. *Summary/Conclusions.* Voreloxin induced durable remissions in 3 dosing schedules. Schedules B and C maintained activity and improved tolerability over A. Schedule C72 is appropriate for further development based on ORR, all-cause mortality, and safety profile.

0071

PERSISTENCE OF CYTOGENETIC ABNORMALITIES AT THE TIME OF COMPLETE REMISSION IN PATIENTS WITH ACUTE MYELOID LEUKEMIA: PROGNOSTIC SIGNIFICANCE AND THE ROLE OF ALLOGENEIC STEM CELL TRANSPLANTATION

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Background. Cytogenetic abnormalities are identified in about 55% of adult patients with acute myeloid leukemia (AML) and are the most important pretreatment factors for predicting the clinical outcome. Prognostic significance of persistent cytogenetic abnormalities at the time of CR and the potential role of allogeneic stem cell transplantation (SCT) in this setting is not well established. *Aims.* To determine the prognostic significance of persistent cytogenetic abnormalities at CR and the role of SCT in this setting. *Methods.* We identified 357 patients with newly diag-

nosed AML (excluding acute promyelocytic leukemia) with cytogenetic abnormalities at initial diagnosis who achieved CR after receiving induction on various frontline protocols between January 2000 and February 2009 at UTMDACC. Among these, 254 patients had a successful bone marrow cytogenetic analysis performed at CR and are the subject of this analysis. We compared the outcome of 183 (72%) patients with normal cytogenetics at CR with 71 (28%) patients who had abnormal karyotype at CR. Overall, 66 patients received SCT at CR. Among them, 51 (77%) patients had normal, and 15 (23%) had abnormal cytogenetics. Kaplan-Meier and Cox proportional hazards regression model were used for statistical analysis. *Results.* The median age of the 254 patients was 52.5 years (range, 16 to 86 years) and the median follow-up was 21 months (range, 3 to 115 months). Patients with cytogenetic abnormalities at CR had significantly shorter CR duration (CRD) (median 6.6 months; P<0.0001) and overall survival (OS) (11.3; P<0.001) compared to patients with normal cytogenetics at CR (39.6 and 45.5 respectively). In multivariable model, after adjusting for other covariates, persistent cytogenetic abnormalities at CR was an independent predictor for shorter CRD (P<0.0001) and shorter OS (P<0.0001); patients with abnormal cytogenetic at CR were more likely to relapse or die than those with normal cytogenetic (2.97 and 2.51 times, respectively). Among patients with cytogenetic abnormalities at CR, those who underwent SCT had significantly better CRD (P=0.01) and OS (P=0.01). *Conclusions.* The presence of cytogenetically abnormal cells at the time of CR is a predictor of relapse, shorter CR duration and shorter overall survival.

0072

REMISSION INDUCTION CHEMOTHERAPY INCLUDING GEMTUZUMAB OZOGAMICIN OFFERS DURABLE REMISSION IN ELDERLY PATIENTS WITH AML AFTER ALLOGENEIC OR AUTOLOGOUS STEM CELL TRANSPLANTATION

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Background. The incidence of acute myeloid leukemia (AML) increases with age but, the prognosis for these elderly patients is dismal. Recent studies have examined the feasibility and efficacy of autologous stem cell transplantation (ASCT) and of reduced intensity conditioning (RIC) stem cell transplantation (SCT) in this population. One key factor that limits the application of SCT for AML of the elderly is the low CR rate achieved by conventional chemotherapy. To improve CR rates, several novel approaches including the use of gemtuzumab ozogamicin (GO) with or without conventional chemotherapy have been attempted. We previously reported on the effectiveness and safety of GO in combination with attenuated conventional chemotherapy in order to improve CR rates in the elderly patients with AML Over 70% of these patients were found to respond to the regimen with acceptable toxicity, which enabled a considerable portion to become potential candidates for SCT. *Aims.* Few reports have been issued regarding the impact of GO as a remission induction regimen on the outcome of SCT in a homogenous population, such as, in elderly patients with AML in first CR (CR1). Here, we analyzed the results of SCT in elderly patients with AML who achieved CR1 with a GO-containing regimen. *Methods.* We included elderly patients (≥55 years) with AML who were medically fit for chemotherapy and achieved CR1 on our institutional protocol including GO. Patients in RIC-SCT group were given a RIC regimen consisting of fludarabine (30 mg/m^2/d, for 5 days) and busulfan (3.2 mg/kg/d, for 2 days). GVHD prophylaxis was undertaken by administering calcineurin inhibitor plus methotrexate. Patients assigned to ASCT were prepared using our modified TAM regimen, which consisted of fractionated total body irradiation, followed by intermediate-dose AraC, and melphalan. *Results.* A total of 54 patients with AML were treated using our institutional remission induction protocol. Of these, 41 patients (75.9%) achieved CR and 17 patients (41.5%) of the patients received RIC-SCT or ASCT; 9 patients received RIC-SCT, and 8 patients received ASCT. We also arbitrarily divided the patients into CD33-high (≥60%) and low (<60%) groups according to CD33 positivity in BM samples at the time of diagnosis. Fourteen patients were allocated to the CD33-high group. With a median follow up of 40.4 months, probabilities of OS, DFS, RI, and NRM at 3 years for all 17 patients were 58.2%, 47.1%, 42.2%, and 18.1%, respectively. OS and DFS were not statistically different in the two transplant groups (Figure 1). Patients without chronic GVHD demonstrated superior disease free survival. On the other hand, those that developed acute GVHD demonstrated inferior OS and DFS. Patients with low BM CD33 expression showed significantly lower OS and DFS than those with high expression. RI was significantly higher in CD33 low group than in CD33 high group. No difference was observed in NRM according to CD33 expression. *Conclusions.* This study has several implications for SCT in elderly patients with AML. First, it shows that GO can improve survival in elderly patients with AML who receive SCT by improving the CR rate; furthermore, this improvement was more marked in the setting of ASCT. This allows a larger proportion of patients to undergo SCT, which is believed to be a potentially curative treatment modality in this population. Second, we reconfirm the impact of acute and chronic GVHD on NRM and relapse, and therefore, on survival. Third, our findings raise the possibility that BM CD33 expression is an important prognostic factor when GO is used for remission induction.

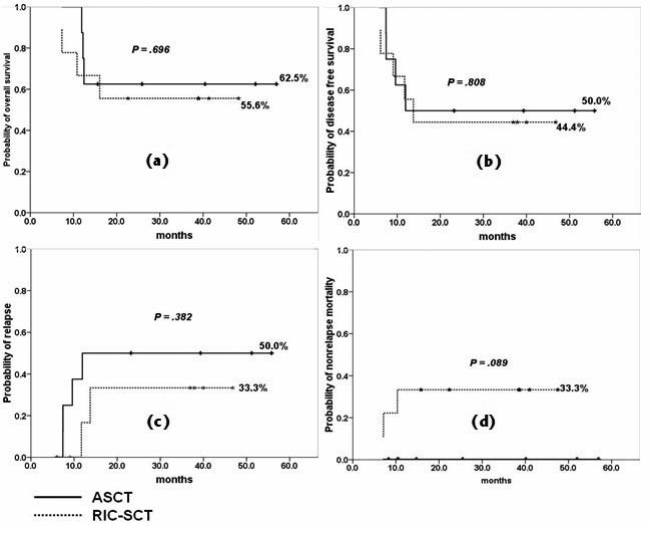

Figure 1. Outcomes according to transplant modalities

0073

PHASE 2 TRIAL OF VORINOSTAT (SAHA) IN COMBINATION WITH GEMTUZUMAB OZOGAMICIN AS INDUCTION AND POST-REMISSION THERAPY IN OLDER PATIENTS WITH PREVIOUSLY UNTREATED ACUTE MYELOID LEUKEMIA (AML)

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Background. Gemtuzumab ozogamicin (GO), an immunoconjugate between a humanized anti-CD33 antibody and a toxic calicheamicin-γ1 derivative, causes DNA damage and apoptosis in AML cells. Preclinical studies have demonstrated that histone deacetylase (HDAC) inhibitors such as vorinostat increase CD33 expression and lower the apoptotic threshold to calicheamicin-γ1. These studies prompted a phase 2 study of vorinostat as chemosensitizer with GO in patients with untreated non-APL AML. *Methods.* After informed consent is obtained, patients receive vorinostat 400 mg orally once daily on days 1-9 and GO 3 mg/m^2 on day 8; hydroxyurea is given to reduce the WBC <10,000/μL before beginning vorinostat. Therapy is repeated for residual disease on day 14, the protocol was amended after 8 enrolled patients to allow a third induction course before response assessment. Patients who achieve either complete remission (CR) or CR with incomplete blood count recovery (CRi) are eligible for 1 cycle of consolidation treatment with vorinostat and GO in the same doses. In patients aged ≥70 years and ECOG performance status (PS) 2-3 (Group 1), the primary outcome of interest is 30-day survival while the primary objective in other patients (Group 2) is determination of the CR/CRi rate after induction therapy. The statistical design monitors CR/CRi rate separately in Group 2 patients with normal or favorable cytogenetics (Group 2A) and other cytogenetics (Group 2B) while allowing results from Group 2A to affect stopping Group 2B and vice versa. In Group 2A, stopping would occur if the probability was >97% that the true CR/CRi rate was <45% while in Group 2B, the criterion probability was 98% and the reference CR/CRi rate was <30%. The 97% and 98% cut-offs were chosen so that there would be no more than 10% probability of early

stopping if the true CR/CRi rates were at least 45% or 30% in Group 2A or 2B, respectively. *Results.* 25 patients, median age 73 (range, 61-80) years, median PS 1.5 (range, 0-3), 60% with secondary AML, have been enrolled: 9 in Group 1 (including 1 with favorable cytogenetics), 8 in Group 2A, and 8 in Group 2B. Among 25 evaluable patients, CR/CRi rates are 1/9 in Group 1, 3/8 in group 2A (2 patients negative for minimal residual disease by flow cytometry), and 0/8 in Group 2B. 1 patient died before day 30 from sepsis in Group 1 and Group 2B. Among the 4 patients with CR/CRi (all CR as best response), 2 received further consolidation therapy with high-dose cytarabine, and 1 patients is undergoing allogeneic stem cell transplantation. Remissions are ongoing at 1, 3, 7 and 8 months. Treatment has been well tolerated with grade 3 hypertension (2 cases) the only notable toxicity besides cytopenias, infections, and infusion-related reactions. Given the above CR/CRi rates and pre-specified stopping rules, the study has closed to accrual in Group 2B while continuing to accrue in Group 1 and Group 2A (registered at ClinicalTrials.gov as NCT00673153). *Conclusions.* Vorinostat in combination with GO has anti-AML activity that may mostly be confined to patients with normal karyotype AML.

0074

A SCORING SYSTEM INCLUDING MOLECULAR GENOTYPE TO PREDICT THE ACHIEVEMENT OF COMPLETE REMISSION IN PATIENTS WITH PRIMARY ACUTE MYELOID LEUKEMIA

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Aim. To analyze the prognostic impact of molecular genotype on the achievement of complete remission (CR), in patients with primary (*de novo*) AML (M3 excluded) *Patients and Methods.* Between december-2003 and November-2009, 606 patients up to 70 years-old were included in the CETLAM AML-03 protocol. Induction therapy consisted in one or two courses of idarubicin, intermediate dose ara-C and etoposide, in addition to G-CSF priming from day 0. Cytogenetics classification was as in the MRC studies: Favorable prognosis (FP), intermediate (IP) and adverse (AP). In the IP group, molecular analysis of NPM1 (NPM1+) mutations, CEBPA mutations and internal tandem duplication of FLT3 gene (ITD/FLT3) was performed. In the AP group, the absence (MK-) or presence of monosomal karyotype (MK+) was studied; MK+ was defined as 2 or more autosomal monosomies, or one monosomy and >1 structural alteration. *Results.* Median age of the series was 53 years (range, 18-73). In 543 (89%) patients cytogenetic data were available; of them, 68 (13%) had FP, 391 (72%) IP (included 248 with normal cytogenetics) and 84 (15%) AP. In the FP group or CBF leukemia, 35 (51%) had the AML1/ETO fusion and 33 (49%) the CBF/MYH11. In the IP group, 72 patients (18%) were $NPM1^+$/FLT3w, 102 (26%) were DIT/$FLT3^+$, 18 (5%) were $CEBPA^+$. The remaining 100 (25%) patients had incomplete molecular information. In patients with AP, 42 were MK- and 31 MK^+. Overall, 457 (77%) of 600 patients evaluable achieved a CR. The rate of CR according to cytogenetics was: FP 91%, IP 77% and AP 70%, P=0.01. In AML with favourable genotype that includes CBF leukaemia, $NPM1^+$/$FLT3^-$ and $CEBPA^+$/$FLT3^-$, RC rates ranged from 91-94% and of note no refractoriness was observed. In patients with AP, CR rate was 76% if MK^- and 63% if MK+ (P=0.48). Multivariate analysis showed that favourable genotype and WBC $<50\times10^9$/L had a positive impact for CR. According to these factors we developed a predictive score, low-risk: favourable genotype and WBC $<50\times10^9$/L, intermediate-risk (1 factor), and high-risk, non favourable genotype and $>50\times10^9$/L with a significant CR rate, 94%, 79%, and 60% respectively, P<0.0001). Moreover, in the favourable genotype those with <50 years-old and WBC $<50\times10^9$/L had a CR rate of 97% vs 69% for those >50 years-old and WBC $>50\times10^9$/L vs 93% with the remaining combinations, P=0.01. *Summary.* Genetic characterization is nowadays mandatory in AML. CR rate is high and refractoriness exceptional in the presence of CBF leukaemia, or NPM1 or CEBPA mutations without DIT/FLT3. WBC is an additional prognostic factor. Using the above score, CR rate can be predicted accurately. Furthermore, in favourable genotype the combination of age and WBC could separate 3 groups of patients with different CR rates. *Supported in part by grants: GR1-01075, ECO07/90065, PI080672 and RD06/0020/0101.*

0075

INVASIVE ASPERGILLOSIS: A NEW IMPORTANT RISK FACTOR ON SHORT AND LONG-TERM SURVIVAL OF AML PATIENTS

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Background. Invasive aspergillosis (IA) remains an important cause of death for acute myeloid leukemia (AML) patients. Recently a lot of efficient innovative agents have been proposed as curative, empiric and more recently prophylaxis therapy in the IFI setting. It has been demonstrated that some agents used either as early treatment or prophylaxis of IFI had a significant impact on early survival (Day 100) of AML patients. *Objectives*. To study the impact of IA incidence during induction therapy for AML patients on short and long term overall survival. *Methods.* A retrospective cohort study was performed in the Hematology department of the Edouard Herriot Hospital, Lyon (France). Patients with newly diagnosed AML between 01/01/2004 and 31/12/2007 were retrospectively included and the follow-up was censored at 30/06/2009. Data were extracted from medical charts and from the prospective surveillance of IA (EORTC diagnosis criteria). The patients with IA after post induction evaluation were excluded (N=5). A Cox proportional hazard model with diagnosis of IA and post induction evaluation (complete remission [CR] of AML or failure of chemotherapy) as the main exposure besides age, year of inclusion, WHO status, cytogenetic group, kind of induction chemotherapy, and hematopoietic stem cell transplantation, was fitted. *Results.* Overall, 262 patients counting for 149 370 patient-days were analysed, the median age at diagnosis was 56.6 years (47.9-64.2 years), and 196 (75%) had CR. There were 58 (22%) IA cases with a median interval between induction and IA of 30 days (range, 16-27 days); 29 (50%) IA were possible, 24 (41%) probable, and 5 (9%) proven. At the last follow-up, 165 (63%) patients died with a median overall survival of 18 months (95% confidence interval [95% CI] 14-23 months). The 4 year-survival of patients having had IA was 14%, and without IA 32% (P=0.01). The 2 year-survival of patients achieving of CR was 54% vs. 5% for patients with failure of chemotherapy was 5% (P<0.001). Cox multivariate analysis showed that patients in CR with IA presented a higher risk of death compared to patients in CR without IA (Hazard ratio=1.66, 95% CI 1.05-2.65, P=0.031). In addition, IA was associated with a higher risk of death in patients with failure of chemotherapy compared to patients in CR without IA (Hazard ratio=6.43, 95% CI 3.72-11.10, P<0.001). The WHO status, cytogenetic group and kind of induction chemotherapy were associated with lower survival.

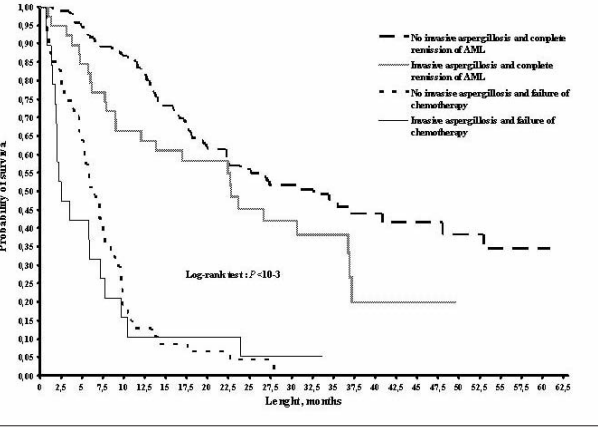

Figure.

Conclusions. IA was associated with a high risk of death in AML patients whether they were in CR or failure after induction chemotherapy. Cytogenetic group or WHO status are not modifiable risk factors for death in this population while prevention of IA with environmental procedures or using individual prophylaxis will improve survival outcome.

0076

A PROGNOSTIC AND PREDICTIVE CLASSIFICATION FOR SURVIVAL AFTER COMPLETE REMISSION IN INDUCTION THERAPY OF ACUTE MYELOID LEUKEMIA

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Background. In the AML 96 study of the Study Alliance Leukemia (SAL), 586 patients with *de novo* or secondary acute myeloid leukemia (AML) and no karyotype t(8;21) achieved complete remission (CR) after two cycles of induction therapy. *Aims.* For these 586 patients, we sought to identify parameters evaluated at diagnosis which provide prognostic information with respect to overall survival. In addition, the predictive value of these parameters was investigated within each of the specific post-remission therapies comprising allogeneic hematopoietic stem cell transplantation, chemotherapy with high-dose cytarabine, or autologous stem cell transplantation (autologous SCT). *Methods.* Candidate variables were diagnosis (*de novo*, post-myelodysplastic syndrome, or treatment-induced secondary AML), age at diagnosis, sex, cytogenetic risk, leukocyte count, platelet count, FLT3-ITD mutant-to-wild-type ratio, NPM1 and CEBPA mutation status, and the percentages of CD34 expression, POX-positive blasts, and blasts in bone marrow at day 15 after start of induction therapy. In contrast to a first approach, continuous variables were not categorized. Instead, fractional polynomials in the multiple (stratified) Cox regression modeling were allowed. Stratification was necessary for the general prognostic model to account for differences in survival probabilities between the possible post-remission therapies. The resulting linear predictor of a model was categorized into risk groups to aid medical decision making. *Results.* At first, the introduction of a new (binary) risk group variable proved to be appropriate: Unfavorable risk was defined by either poor cytogenetic risk or treatment-induced AML. All other patients were considered to have standard risk. The final multiple model comprised the variables age, new risk group, FLT3-ITD mutant-to-wild-type ratio, and the percentages of CD34 expression (all P<0.005). Of 463 patients with data on these variables, 235 (51%) had died. The linear predictor of the final model was categorized into three risk groups with significantly different survival probabilities (P<0.0001). The low-risk group was composed of 201 patients (71 died) with a four-year survival probability of 0.65. The same number of patients (112 died) was allocated to the intermediate-risk group. The four-year survival probability was 0.44. The 61 patients of the high-risk group (52 died) had a four-year survival probability of 0.12. Also within each of the post-remission therapies, the three risk groups were able to predict patient groups with significantly different survival probabilities (all P<0.0005). In addition, in each risk group it was possible to identify a treatment which appears to be the most promising and which was not the same for all risk groups. *Conclusions.* Four variables were identified as independent prognostic factors for survival after CR in induction therapy. The corresponding prognostic score resulted in three risk groups also with predictive survival differentiation within each of the post-remission therapies. The treatment with the best survival probabilities differed between risk groups. Hence, the prognostic and predictive classification supports risk-adapted treatment and provided new hypotheses for treatment optimization.

0077

FINAL RESULTS OF A PHASE 2 PHARMACOKINETIC/PHARMACODYNAMIC (PK/PD) STUDY OF COMBINATION VORELOXIN AND CYTARABINE IN PATIENTS WITH RELAPSED OR REFRACTORY ACUTE MYELOID LEUKEMIA

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Background. Voreloxin (VOR), an anticancer quinolone derivative that inhibits topoisomerase II, is active in ovarian cancer and acute myeloid leukemia (AML). Nonclinical studies showed synergistic activity of VOR and cytarabine (ara-C) (Scatena Cancer Chemother Pharmacol 2010). *Aims.* Assess clinical activity and safety/tolerability of combination VOR/ara C treatment; characterize PK/PD profile. *Methods.* Phase (ph) 1b results were presented previously (Proc ASCO 2009). Ph 2 expansions in first relapse (frel) and primary refractory (pref) AML were at 80 mg/m^2 VOR/400 mg/m^2 ara-C by 24-hr CIV in schedule A (A) and 90 mg/m^2 VOR/1 g/m^2 ara-C by 2-hr IV infusion in schedule B (B). VOR dosing days 1, 4; ara-C daily ×5. Up to 4 cycles allowed. Response determined by IWG criteria. Blood, urine, and bone marrow aspirate (BMA) collected for PK or PD analysis. Ph 2 populations pooled and subset analysis of pref/early frel (efrel) outcome performed as safety/efficacy results were comparable in A and B. All patients provided informed consent. *Results.* CR+CRp+CRi (ORR) was 32% (12/37) frel and 25% (8/32) pref; most were CR (17 of 20). Fourteen ph 2 patients went to transplant (12 responders, 1 partial responder, 1 treatment failure). As of a data cutoff of 8 Jan 2010, preliminary median overall survival (OS) was 7.8 months (mo) (95% CI 4.7-10.2). All-cause mortality was 3% (2/69) at 30 days and 8% (5/63) at 60 days. Activity was similar in patients with efrel (CR1 ≤12 mo) and pref (ORR 28%, OS 7.3 mo [95% CI 6.0-9.9]), a patient group with poor outcomes. Nonhematologic grade ≥3 adverse events >10% at VOR 70-90 mg/m^2: aggregated (ag) upper GI mucositis 17%; ag lower GI mucositis 9%; febrile neutropenia 37%; ag sepsis/bacteremia 23%, ag infections 15%, and ag pneumonia 10%; hypokalemia 19%. PK/PD: VOR PK was dose proportional and unaffected by ara-C. DNA damage PD response seen with increased pDNA PKcs/pCHK2 in BMA of 15/23 patients at ≥34 mg/m^2 VOR/ara-C. *Summary/Conclusions.* Activity of combination VOR with intermediate dose ara-C is promising, and supports the planned randomized pivotal phase 3 study in relapsed or refractory AML. Safety and activity profile in the subset of pref and efrel (CR1 ≤12 mo) patients: ORR (28%), 30- and 60-day all cause mortality (<10%), and OS (7.3 mo) compares favorably with recent data for other AML therapies (Litzow BJH 2009; Giles Blood 2009).

0078

WT1 EXPRESSION IN PERIPHERAL BLOOD HAS NO PROGNOSTIC VALUE BUT CAN PROVIDE MORE USEFUL INFORMATION ON MRD COMPARED TO BM IN CHILDREN WITH AML

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Background. The Wilms' tumor gene 1 (WT1) is highly expressed in a large proportion of human acute leukaemias and other haematological malignancies. In the majority of studies on childhood AML, the WT1 detection is realized by analysis of bone marrow samples (BM), although there are some suggestions that peripheral blood (PB) is more suitable for this purpose, especially in adult AML. *Aims.* To investigate the correlation between WT1 levels in BM and PB, we analyzed 90 patients with *de novo* childhood AML. Normal PB samples and normal or regenerating MRD-negative BM samples were used as controls to define the range of physiological WT1 levels. *Methods.* RQ-PCR for absolute quantification of total WT1 was designed according to Europe Against Cancer Program. *Results.* We detected the WT1 expression in 92% of BM and 91% of PB samples. Physiological expression of WT1 in unsorted BM and PB of healthy donors samples was 29 and 1.4 WT1/ABL×10^4 NCN (range 22-115 and 0.2-13.2), respectively. WT1 overexpression (defined as >1.5-fold higher than maximum of physiological level) was found in 75% of BM and 80% of PB samples. The median of WT1 level in BM was 0.2 log higher in comparison with PB (P=0.01) and we found an excellent correlation between the values detected in BM and PB (correlation coefficient 0.89, P<0.0001). There was no relation between WT1 levels in PB or BM and the percentage of blasts. The difference in WT1 expression between patients' samples and healthy controls was 2.0 log for BM and 3.1 log for PB. Overall, we found significantly higher WT1 expression in childhood AML than in ALL (P<0.001 for BM, P<0.0001 for PB). According to FAB classification, M3 was associated with the highest WT1 (P<0.0001 for both BM and PB) and patients with M5 AML had the lowest WT1 expression (P<0.0001 for both BM and PB). Standard risk patients expressed higher WT1 than high risk patients (P<0.0001 for both BM and PB). We did not find any correlation between WT1 level and age, sex, d15 treatment response and relapse-free or overall survival. *Conclusions.* In contrast to studies in mostly adult AML, we demonstrate no prognostic significance of WT1 expression at diagnosis of paediatric AML. Based on this representative group of patients we can conclude that PB samples are suitable for detection of WT1 expression. Moreover, the more significant difference between WT1 level detected in patients' samples and the level found in normal PB suggests that the peripheral blood can

provide more useful information on MRD in childhood AML.

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0079

THE PRESENCE OF P-GLYCOPROTEIN (MDR1) AFFECTS THE ABILITY OF AML PATIENTS TO ACHIEVE COMPLETE REMISSION; RESULTS OF A METAANALYSIS OF THE LITERATURE

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Background and Aims. The expression of P-gp is highly prevalent in acute myeloid leukemia (AML). Current treatment options, including anthracyclines and anthracenediones, are substrates for P-gp. A meta-analysis was carried out to evaluate the influence of P-gp on treatment outcome in AML. *Methods.* Studies investigating P-gp (function and/or protein and/or mRNA) in AML and reporting the relevant outcomes (in particular, complete remission [CR]) were considered for this meta analysis. Only patients diagnosed with AML, and who either proceeded to treatment with anthracycline drugs or mitoxantrone, or who had previously been treated with anthracyclines or mitoxantrone, were eligible to be included in the meta-analysis. The primary analysis population for the meta-analysis was the Evaluable Population (EP), that is, all patients from included studies whose P-gp status was known. Studies for inclusion in the meta-analysis were identified in a thorough literature search, including the PubMed database, using a pre-specified search algorithm. The incidence of P-gp was tabulated by publication and overall. The odds ratio (OD) for CR (positive versus negative P-gp status) was tabulated by publication with 95% confidence interval (CI). *Results.* 74 studies were included in the analysis. Odds ratios could be calculated from 55 studies which provided 4549 patients evaluable for both CR and P-gp status. The median CR rate across all studies was 65%; CR rates were 78% in Pgp- and 47% in Pgp+ patients. Of all 55 publications, 49 gave OD<1, including 31 with statistical significance. Of the 6 publications which gave OD>1, none reached statistical significance. The overall odds-ratio for CR (positive versus negative P-gp status) was 0.257 (95% CI, 0.200-0.329). *Conclusions.* CR is much less likely to occur in patients with P-gp expression. These patients should be considered for treatment options that are unaffected by P-gp.

Cellular and molecular hematology 1

0080

IN ADDITION TO GRANULOPOIESIS, HUMAN BONE MARROW ADIPOCYTES INHIBIT ALSO ERYTHROPOIESIS THROUGH CELL-CELL CONTACT BUT INDEPENDENTLY OF NEUROPILIN-1

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Background. Fat cells are heterogeneously present in the bone marrow (BM) and replace hematopoietic cells in BM failure disorders such as aplastic anemia. There is growing evidences showing that bone marrow adipocytes play an active role in the regulation of hematopoiesis. Adipocytes are the main stromal cells in femoral bone marrow. With age, their number increases in the bone marrow cavity, and simultaneously, erythropoiesis and granulopoiesis decrease. In our previous study we demonstrated that human bone marrow adipocytes block granulopoiesis through inhibition of G-CSF production by a mechanism involving direct cell-cell contact and neuropilin-1. *Aim.* The aim of this study was to investigate whether or not adipocytes inhibit erythropoiesis and if so by which mechanism. Material and *Methods.* Adipocytes and $CD34^+$ were isolated from femoral bone marrow and iliac crest aspirate obtained from patients undergoing hip surgery. The ethical committee and donor's consents are obtained. Adipocytes and $CD34^+$ were co-cultured either in direct cell-cell contact or in absence of cell contact using 0.4 µm transwell chamber leading only to culture medium exchanges. The effect of adipocytes on $CD34^+$ differentiation is assessed in the absence of exogenous cytokine except for erythropoietin (Epo) (2UI/mL) for erythropoiesis. Neuropilin-1 was neutralized by an increased amount of a polyclonal antibody (0-20 µg/mL). Co-cultures were stopped after one to five weeks. Medium were collected for cytokine production by ELISA analysis, and erythropoiesis was evaluated by May-Grünwald Giemsa staining of cytospin and glycophorin-A (GPA) detection by flow cytometry analysis. Experiments were also performed *in vivo* by engrafting $CD34^+$ and adipocytes into the kidney capsule of NOD/SCID mice. *Results.* Analysis of *in vitro* co-cultures and *in vivo* transplantation of Adipocyte and $CD34^+$ cells in NOD/SCID mice led to macrophages (Mo) and dendritic cells (DC) differentiation. Likewise, Adipocytes produced SCF, M-CSF and GM-CSF. Adipocytes established cell-cell contacts with Mo, which were not critical for their differentiation since in transwell assays M-CSF was produced and Mo were also obtained. In contrast, adipocytes impaired erythopoiesis even in the presence of high dose of Epo whereas granulopoiesis inhibition was restored by the addition of G-CSF. In transwell assays erythropoiesis and granulopoieis concomitantly to G-CSF production were restored. We postulated that NP-1 could be involved in this inhibition since: 1) NP-1 is differentially expressed between hematopoietic iliac crest BM (rich in hematopoietic cell and weakly NP-1 positive) and femoral BM (poor in hematopoietic cells and highly NP-1 positive); 2) the expression of NP-1 increased in adipocytes and $CD34^+$ co-cultures; 3) the role of NP-1 in the modulation of cytokine production including Tpo and Flt-3 ligand by stromal cells previously reported and 4) NP-1 plays a role in various cell-cell contact systems. In adipocytes and $CD34^+$ co-cultures, NP-1 neutralization led to G-CSF production and granulopoiesis restoration but has no impact on erythropoiesis inhibition even in presence of high dose of Epo. *Conclusions.* Bone marrow adipocytes block both erythropoisis and granulopoiesis by two different mechanisms. Full understanding of these mechanisms may provide new targeted therapies in bone marrow failure including aplastic anemia.

0081

PROFILING OF MICRORNA EXPRESSION IN PURIFIED HEMATOPOIETIC POPULATIONS AND IN SINGLE CELLS

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Background. The hematopoietic system is comprised of a large number of highly specialized cell types that occupy distinct niches and which perform a diversity of functions ranging from innate immune response to oxygen transport. All these cell types are thought to be derived from a common stem cell and represent a hierarchal tree of differentiation. microRNAs (miRNAs) are critical players in orchestrating this differentiation. Due primarily to technical limitations in the analysis of limited cell populations the program of miRNA expression across the hematopoietic tree is largely unknown. *Aims/Methods*. Here we report the development of a microfluidic RT-qPCR approach for global miRNA profiling in limited populations and apply this to expression analysis of 27 distinct cell populations from the murine hematopoietic system. Expression of 288 miRNAs in each population was measured in multiple replicates for a total of over 80,000 RT-qPCR assays using microfluidic qPCR arrays (Fluidigm Biomark™ Dynamic Array). Using synthetic miRNA standards the sensitivity and efficiency of each assay was calibrated to allow for accurate comparison of expression across species and populations. In addition we show this technique is capable of measuring up to 12 miRNA species at single-cell resolution. *Results.* We demonstrate that global miRNA profiling of the murine hematopoietic tree allows for direct and independent reconstruction of the known hierarchal relationships. We further find that the number of miRNA species expressed in a cell population is not correlated with differentiation state and that miRNA expression patterns in stem cell and progenitor populations are closely related with major reprogramming upon commitment to a single lineage. Single cell measurements further show that miRNA expression levels are tightly regulated within highly purified populations, suggesting that miRNA may be suitable biomarkers for assessing heterogeneity in a given population. *Summary/Conclusions.* Taken together, we show that miRNA expression patterns determine the differentiation state of a hematopoietic cell as well as its cellular identity. We could not identify a stem cell specific miRNA, but multiple differentially expressed miRNAs. Our findings as well as our novel approach to minimize method-induced biases provide the basis to unravel the role of miRNAs in stem cells in particular and hematopoiesis in general.

0082

DOWN-REGULATION OF THE RUNX1-TARGET GENE NR4A3 CONTRIBUTES TO THE PRELEUKEMIC STATE IN FAMILIAL PLATELET DISORDER/ACUTE MYELOGENOUS LEUKEMIA

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RUNX1 (AML1) encodes a DNA binding α-subunit of the core binding factor (CBF), a heterodimeric transcription factor. RUNX1 is a master regulatory gene in hematopoiesis and its disruption, most frequently by chromosomal translocations, is one of the most common aberrations in acute leukemias. Inactivating or dominant-negative mutations in the RUNX1 gene have been also identified, e.g. in pedigrees of familial platelet disorders with a variable propensity to develop acute myeloid leukemia (FPD/AML) 1. We performed a comparative analysis of hematopoietic stem cells in two FPD/AML pedigrees with distinct RUNX1 germ line mutations, i.e. the R139stop in a pedigree without AML and the R174Q mutation in a pedigree with AML. R174Q mutation induced a marked increase in the clonogenic potential of immature $CD34^+CD38^-$ progenitors, which demonstrated some self-renewal capacities, a property that lack R139stop progenitors. This increased proliferation correlated with a profound reduction in the expression of NR4A3, a gene previously involved in leukemia development. Furthermore we demonstrated that NR4A3 was a direct target of RUNX1 and that restoration of NR4A3 expression partially reduced the clonogenic potential of patient progenitors. We propose that the down-regulation of NR4A3 contributes to establishing the preleukemic state in RUNX1 mutated hematopoietic progenitors.

0083

THE MEK/ERK PATHWAY BUT NOT P38MAPK PROMOTES CHROMATIN REMODELING AT RETINOIC ACID TARGET GENE PROMOTERS AND POTENTIATES GRANULOCYTIC DIFFERENTIATION VIA RAR ALPHA RECRUITMENT

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Background. Granulopoiesis is under the control of cytokines and their receptors but also of intracellular transcription factors aimed at regulating specific gene expression along the differentiation. Retinoic Acid regulates the expression of target genes through the binding of its specific receptors (RARs) and the recruitment of Histone Acetyl transferases (HAT) such as CPB/P300 on the promoter of these genes leading to chromatin remodelling. The presence of the PML-RARα fusion protein in Acute Promyelocytic Leukemia (APL) cells leads to differentiation blockade which can be overcome by therapeutic concentrations of ATRA. However, ATRA resistance may occur in case of mutations in the ligand binding domain of PML-RARα as in the UF-1 cell line. *Aims.* We took advantage of the UF-1 cell line RA resistant model to analyse the potential role of cytokine activated signalling pathways such as MAP Kinases to identify key steps that could enhance RA induced granulocytic differentiation. *Methods.* RA resistant UF-1 and RA responsive NB4 cells were used. Differentiation was studied by CD11b and CD11c expression and by oxydo reduction analysis using the NBT test. RA target genes expression was analysed by qRT-PCR. Presence of RA receptors, HAT proteins and acetylated histones on the promoter regions of RA target genes was analysed by ChIP experiments. *Results.* UF-1 cells treated with G-CSF and RA but not G-CSF alone were able to differentiate similarly to the NB4 cells treated by RA alone. G-CSF allowed to restore RA transcriptional activity as the expression of RA target gene such as RARα2 and RARβ2 was reinduced. Also, the recruitment of RARα, CBP/P300 and acetylated histones H3 and H4 in RA activated promoter regions were restored in UF-1 cells 1 hour after double treatment to a level similar to NB4 cells treated by RA alone. After RA treatment we observed in NB4 cells, but also in UF-1 cells, an early activation of the p38MAPK pathway concomitant of PML-RARα and RARα phosphorylation. It is likely that p38MAPK response is not altered in RA resistant cells. Furthermore, G-CSF did not modify the p38MAPK response to RA in UF-1 cells. Then we focused on the MEK-ERK1/2 pathway because G-CSF induced a strong phosphorylation of p42/p44 ERK1/2 and the specific MEK inhibitor UO126 completely abrogated the differentiation of UF-1 cells treated by G-CSF and RA. This was confirmed using siRNA directed to p42 and p44. The MEK-ERK pathway was involved in the regulation of RA transcriptional activity as a pretreatment by UO126 totally abolished the reinduction of RA target genes by the double treatment. Furthermore, the RA + G-CSF induced recruitments of the transcription factor RARα CBP/P300 and acetylated histones H3 and H4 at RA target genes promoter were strongly antagonised. *Conclusions.* These results demonstrate that specific cytokine activated pathways can participate in the regulation of nuclear receptor activity and chromatin remodelling. We identified the MEK-ERK pathway as specifically involved in the enhancement of RA induced granulocytic differentiation. This represents a new potential target to extend differentiating therapy to RA resistant AML.

0084

GATA-1 MEDIATES PIGM TRANSCRIPTION IN IGD AND NORMAL ERYTHROID TISSUES

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Background. Inherited glycosylphosphatidylinositol (GPI) deficiency (IGD) is characterised by thrombosis and epilepsy but minimal hemol-

ysis; by contrast, intravascular hemolysis is a major feature of acquired GPI deficiency, i.e., paroxysmal nocturnal hemoglobinuria. IGD is caused by a -270C>G mutation that disrupts binding of the transcription factor (TF) Sp1 to the core promoter of the mannosyltransferase-encoding gene PIGM. In IGD patients, GPI expression in blood cells is variable: for example, while GPI synthesis in granulocytes is severely deficient, it is nearly normal in erythrocytes, explaining thus the absence of significant intravascular hemolysis in IGD and suggesting an erythroid-specific transcriptional regulation of PIGM. *Aims.* We tested the hypothesis that the erythroid/megakaryocytic-specific TF GATA-1 is a critical regulator of PIGM transcription and can rescue GPI biosynthesis even in the presence of the C>G mutation. *Methods and Results.* Bioinformatic analysis predicted the existence of three GATA-1 binding motifs - S1, S2 and S3- in the promoter of PIGM. Consistent with a role of GATA-1 in the transcriptional regulation of PIGM, promoter activity as assessed by reporter assays in which luciferase activity is driven by a 2Kb wild type (WT) PIGM promoter construct was higher in the erythroid K562 than in the myeloid HL60 cells. Reporter activity was proportionally increased for both a 2Kb (WT) or c>g mutated (Mut) construct when co-transfected with GATA-1. This effect was abrogated when shorter constructs not containing the predicted GATA-1 motifs were used, suggesting GATA-1 acts through these distally located motifs. In support of this, reporter assays using promoters with the predicted motifs inactivated individually or in combination suggested that all 3 GATA-1 motifs are active; more so the proximal S1 motif which supported transcription even in the presence of the c>g mutation. By contrast, the distal S2 &S3 motifs appeared to require an intact Sp1 site to activate transcription. Taken together these data suggest that GATA-1 acts on the PIGM promoter through Sp1-dependent and -independent mechanisms. The functional significance of S1-3 motifs as GATA-1 binding sites was also confirmed *in vivo* by chromatin immunoprecipitation (ChIP) assays in K562 cells. Further underpinning its functional significance as an important transcriptional regulator of PIGM in erythroid cells, reduction of GATA-1 protein levels by 70% in K562 cells using siRNA was associated with a 30% reduction in PIGM mRNA as compared to scramble control while enforced expression of GATA-1 in the GATA-1-negative patient B cells led to a partial correction of the GPI deficient phenotype as assessed by flow cytometry. *Conclusions.* These results indicate that GATA-1 is critical for the tissue-specific transcriptional control of PIGM in erythroid cells and it can support sufficient transcriptional activity even of the mutated PIGM promoter explaining thus the absence of significant haemolysis in IGD.

0085

THE EVI1 TRANSCRIPTION FACTOR REPRESSES ITS OWN TRANSCRIPTION IN HUMAN CELLS

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The EVI1 gene (3q26) codes for a transcription factor with important roles in development and leukemogenesis. At present, 3q26 rearrangements are the only known mechanism that leads to EVI1 overexpression; however, transcriptional activation of this gene has been reported in about 9-20% AML patients with no 3q26 abnormalities, and is also associated with an unfavorable outcome. Our aim was to analyze the promoter region of EVI1 to look for transcription factors that could regulate its transcription. *Material and Methods.* Bioinformatic analysis to identify hypothetical transcription factors binding sites in the genome, and validation by chromatin immunoprecipitation (ChIP) and luciferase assays. *Results.* Bioinformatic analysis within the 3,000bp upstream region of the TSS of the EVI1 gene using MATCH and TFSEARCH softwares identified several transcription factors involved in hematopoiesis, including Evi1. This result would suggest the possibility of Evi1 being a regulator of its own expression. ChIP assays confirmed that Evi1 binds to 3 of the 8 DNA predicted sites. To identify if Evi1 is directly responsible for its promoter activity, we generated seven constructs comprising different promoter regions. Cotransfection with EVI1 showed reduced luciferase activity in a dose-dependent way, suggesting that Evi1 acts as a repressor transcription factor of its own expression. To find out whether Evi1 itself regulates its transcription or other factors were required, we produced promoter constructs carrying mutations in a single or in several EVI1 binding sites. None of these constructs affected the luciferase activity. In spite of the results, the dose-dependent effect observed prompted us to investigate the putative cooperation between different regions among the promoter. We generated serial deletions in the reporter construct carrying different EVI1-binding sites. This approach allowed us to identify a functional region of 318pb in the proximal promoter region of EVI1, where there are several binding sites for transcription factors with important roles in hematopoiesis. Taken together, these results would suggest that Evi1 represses its own expression, probably through the interaction with other proteins. Further studies are in progress to identify these putative transcription factors.

0086

THE EFFECT OF NILOTINIB AND DASATINIB ON TELOMERASE ACTIVITY AND REGULATION IN K562 CELLS

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Background. The cytogenetic hallmark of chronic myeloid leukemia (CML) is the t[9;22] [q34;q11] translocation creating the BCR-ABL oncogene encoding for the BCR-ABL tyrosine kinase oncoprotein. BCR-ABL activity leads to uncontrolled cell proliferation and reduced apoptosis, resulting in malignant expansion of bone marrow stem cells. Since CML is caused by this distinct genetic lesion, it was possible to design an effective targeted molecular therapy. Imatinib is a tyrosine kinase inhibitor selective for BCR-ABL indicated for treatment of CML. Despite significant hematologic and cytogenetic responses, resistance to imatinib may occur. In order to override resistance, 2nd-generation tyrosine kinase inhibitors (TKIs), such as nilotinib and dasatinib, were developed. The clinical activity of imatinib, nilotinib and dasatinib is attributed mainly to their kinase inhibitory effect. However, we have recently shown that imatinib significantly inhibits telomerase activity in K562 cells sensitive and resistant to imatinib and in CML patients. We further showed that telomerase inhibition in these cells is most likely due to downregulation of the AKT-signal transduction cascade (Exp Hematol 38:27-37, 2010). *Aims.* We hypothesize that similar to imatinib, the new TKIs target telomerase and the signaling pathways upstream of it. Our goals are to study the effects of nilotinib and dasatinib on telomerase activity and regulation at the transcriptional and post-translational levels in $BCR^{-}ABL^{+}$ (K562) and BCR-ABL- (HL60) myeloid cell lines and in CML patients. *Methods.* The effect of nilotinib and dasatinib on cell survival is assessed by WST-1 proliferation and FACS assays. Telomerase activity following exposure of the cells to the new TKIs is evaluated by the TRAP assay. The effect of these agents on telomerase regulation is measured by real-time PCR, Western blot, nuclear and cytoplasmic fractionation and chromatin immunoprecipitation assays. *Results.* We show that similar to imatinib, the new TKIs target telomerase and reduce its activity by ~90% in $BCR\text{-}ABL^{+}$ (K562) cells. Nilotinib and dasatinib also caused a reduction in telomerase activity in the $BCR\text{-}ABL^{-}$ cell line, HL60. We show that these agents exert their effect on telomerase in an indirect manner. Telomerase regulation involves transcriptional and post-translational steps. At the transcriptional level: The promoter of the catalytic subunit of telomerase, hTERT, contains Sp1 binding sites. Phosphorylated-Sp1 activates the hTERT promoter. At the post-translational level: Telomerase is active when phosphorylated by AKT, and inactive when dephosphorylated by protein phosphatase 2A (PP2A). We demonstrate that the reduction observed in telomerase activity is a result of transcriptional (90% reduction in hTERT mRNA expression) and post-translational (hTERT phosphorylation) modifications generated by these TKIs. *Summary/Conclusions.* Since telomerase is activated in ~90% of human tumors, it has become an extremely attractive target for new anticancer interventions. Our results demonstrate the ability of imatinib, nilotinib and dasatinib to inhibit telomerase activity in $BCR\text{-}ABL^{+}$ as well as in BCR-ABL- cell lines, implying that these agents inhibit telomerase activity in a BCR-ABL independent manner. Thus, this study shows that cells, known to be resistant to these agents with regards to their effect on BCR-ABL, could still be sensitive to their effect on other cellular components such as telomerase.

0087

NOVEL MUTATIONS AT THE TNFRSF6 GENE IN SPANISH PATIENTS WITH AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME (ALPS)

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Background. The autoimmune lymphoproliferative syndrome (ALPS) is a genetic disorder of lymphocyte apoptosis. ALPS is characterized by childhood onset of lymphadenopathy, hepatosplenomegaly, autoimmune cytopenias, elevated (>1.5%) double negative T (DNT; CD3$^+$, TCRalpha-beta+, CD4-, CD8-) lymphocytes in peripheral blood and an increased risk of lymphoma. Most cases (>65%), known as ALPS Type Ia, are associated with dominant heterozygous germline mutations in the gene TNFRSF6 encoding the protein for CD95 (Fas). *Methods.* We studied 32 individuals from different Spanish Hospitals. Selected ALPS patients meet Clinical (lymphadenopathy, splenomegaly and/or cytopenias) and Laboratory (hypergammaglobulinemia and elevated DN T cells) criteria. TNFRSF6 gene was analized by DNA sequencing in all the patients. If there was found a splicing mutation, cDNA was also analised. In selected cases, where no TNFRSF6 mutation was found, TNFSF6 (Fas ligand) and CASP-10 (caspase-10) genes were evaluated by sequencing. *Results.* 26 patients (81%) were found to be heterozygous for a single TNFRSF6 mutation (ALPS Ia). Seven different alterations were identified affecting exons 2, 8 and 9. Four of these mutations were not previously described. The mutations include insertions, splicing defects, missense and nonsense mutations. The other 6 patients do not have any mutation in TNFRSF6, TNFSF6 or CASP-10 gene. Hence, they were classified as ALPS III. As compared to controls and healthy relatives bearing the same genetic alterations, ALPS Ia patients demonstrated significantly higher IL-10 and IgE serum levels. Those markers were also elevated in ALPS III patients but on a much less remarkable fashion, with one exception. That later case is a female child with ALPS and a severe clinical presentation, with very high IL-10, IgG, IgA and IgE levels. In this ALPS patient, mutations in TNFRSF6, TNFSF6, CASP-8, CASP-10, N-Ras and FADD genes have been excluded. *Conclusions.* The combination of elevated DNT counts (>1.5%) with increased IL10 and IgE serum levels in blood is strongly linked to the presence of a FAS mutation. This enables the targeting of patients with clinical features of ALPS to have a more directed evaluation particularly in regard to DNA sequencing for FAS mutations, and other apoptosis-related genes. Several novel FAS mutations were found in the Spanish patients.

0088

BCR-ABL INDUCED TRANSFORMATION UNCOVERS A CRITICAL ROLE OF C-JUN IN VEGF REGULATION

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Background. Dysregulation of AP-1 transcription factors is a frequent event in human cancer. The loss of the AP-1 family member JunB has been observed in human myeloid and B lymphoid leukaemia and is associated with a poor prognosis. In contrast, c-Jun is frequently overexpressed in human cancer and is considered a proto-oncogene. In particular, c-Jun has been shown to be involved in p53 mediated apoptosis. *Aim.* In this study, we have investigated the role of c-Jun for p185BCR-ABL induced leukemia. *Methods.* We therefore worked with p185BCR-ABL transformed c-junΔ/Δ CD19-CRE cell lines. To analyze tumor formation, we injected leukemic cells subcutaneously and intravenously into immuno-compromised mice. We used a pMSCV-c-Jun-puro based retrovirus and a murine VEGF-A construct to determine the interplay of these two proteins in leukemia/lymphoma. *Results.* Whereas previous studies have shown that the loss of JunB in leukemic cells accelerates disease progression, we could show that the lack of c-Jun is associated with increased disease latency. Both, leukemia and lymphoma formation is delayed. Interestingly, c-Jun deficient p185BCR-ABL transformed cells were not capable to form lymphomas over a certain size. Histological analysis of the tumor tissue revealed a drastically reduced vessel density in c-Jun deficient lymphomas accompanied by a central necrosis of the tumors. Accordingly, real time PCR experiments showed decreased VEGF levels in c-junΔ/Δ tumor cells when compared to control cells. Re-expression of VEGF in c-Jun deficient p185BCR-ABL transformed cells partially reverted the phenotype and resulted in an increased tumor size. *Conclusions.* Thereby our study identified c-Jun as a key regulator of VEGF synthesis in p185BCR-ABL transformed cells.

0089

WITHDRAWN BY THE AUTHORS

0090

NITRIC OXIDE PRODUCTION BY BONE MARROW STROMAL CELLS REGULATES HEMATOPOIETIC PROGENITOR CELL SURVIVAL THROUGH ADHESIVE INTERACTIONS

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Nitric oxide (NO) is a small gaseous molecule with diverse roles including the regulation of cell proliferation, differentiation, apoptosis, adhesion and migration. NO is derived from L-arginine by the nitric oxide synthase (NOS) family of enzymes. At least three distinct NOS isoforms have been identified in mammalian cells including the endothelial (eNOS), neuronal (nNOS) and inducible (iNOS). Recently, we have shown that NO donors induce CXCR4 expression in human CD34 positive cells suggesting that NO production may regulate the migration and adhesion of hematopoietic progenitors. To determine the relevance of these findings and define the role of NO in the biology of hematopoietic progenitor cells, we first investigated NOS expression in hematopoietic and non-hematopoietic cells. Using quantitative reverse transcription-PCR analysis, we demonstrate that nNOS, and eNOS isoforms are highly expressed in Human Umbilical Vein Endothelial Cells (HUVEC) and osteoblastic cell lines, but there was no or low expression of these NOS in hematopoietic cells. In agreement with these analyses, we found large amounts of nitrite (a stable derivative of NO) in the culture medium of stromal MS5 (a cell line used in laboratories to support hematopoiesis) and osteoblasic (MG-63) cell lines. To determine the biologic effects of NO on hematopoietic progenitor cell survival, cord blood CD34$^+$ cells were cultured on MS5 cells in the presence/absence of NOS inhibitor (L-NAME) for three days, then hematopoietic progenitor numbers were determined by culture in a semi-solid medium and colonies were quantified. Treatment with L-NAME reduced colony number by 33, 42 and 54% with 10, 100 and 500 µM L-NAME concentrations, respectively. This effect is NO specific since the diminution of progenitor number was reverted by adding NO donor in the culture medium. To understand how NO can regulate hematopoiesis, we investigated its effect on CXCR4 and AML1(Runx1) expression. We found that inhibition of NO production by stromal cells results in diminution of CXCR4 and AML1 RNA messenger levels. To determine if the observed NO effects on hematopoiesis are related to adhesion, cultures were performed in transwell plates separating CD34$^+$ cells from MS5 cells. When CD34$^+$ cells were separated from stromal cells, L-NAME treatment did neither affect progenitor number nor AML1 expression. These results indicate that NO is produced by bone marrow stromal cells and regulates the survival of hematopoietic progenitor cells through a contact dependent pathway.

0091

CDK6 AS TUMOR SUPPRESSOR OR PROMOTER - P16INK4A MAKES ALL THE DIFFERENCE

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Background. Small molecular compounds directed against cell cycle kinases are currently developed and tested for their optimal clinical use to treat cancer in people. The kinase Cdk6 is expressed at high levels in lymphoid malignancies. *Aim.* We therefore studied the impact of Cdk6 for p185BCR-ABL and NPM-ALK driven tumorigenesis. *Methods.* We analyzed p185BCR-ABL and NPM-ALK driven lymphoma/leukemia in $Cdk6^{+/+}$ and $Cdk6^{-/-}$ mice. We used a pMSCV-Cdk6-puro based retrovirus to analyze the effect of Cdk6 over-expression in p185BCR-ABL transformed wild type cells *in vivo* and *in vitro*. Chromatin-immunoprecipitation showed the relation between Cdk6 and p16INK4a. The use of a pMSCV-Cdk6R31CΔC-puro and a pMSCV-Cdk6K43M-puro based retrovirus cleared the role of the C-terminus and the kinase activity of Cdk6 for p16INK4a regulation. We analyzed the protein levels of Cdk6 and p16INK4a in human B- and T-cell lymphoma tissue arrays by immunohistochemistry. *Results.* As anticipated, tumor formation was strongly delayed in a $Cdk6^{-/-}$ background due to a reduced proliferation of the transformed lymphoid cells. Surprisingly, a similar outcome was observed upon enforced Cdk6 expression in lymphoid tumor cells. p185BCR-ABL driven lymphoma/leukemia induced with decreased incidence and increased latency. This unexpected tumor suppressing property of Cdk6 was accounted to the finding that Cdk6 bound to the promoter of the tumor suppressor, p16INK4a and caused its expression in a kinase independent fashion. Studies of human B- and T-cell lymphomas confirmed an inverse relationship between Cdk6 and p16INK4a. *Summary/Conclusions.* Hence, a tumor promoting effect of elevated Cdk6 levels - as observed in lymphoid malignancies - required a disrupted p16INK4a expression. This insight is of major importance to anticipate and predict which patients will benefit from a treatment interfering with Cdk6.

0092

ERYTHROPOIETIC EFFECTS OF IL-17: INVOLVEMENT OF MAPKS AND GATA TRANSCRIPTION FACTORS

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Background. Interleukin (IL)-17 is the founding member of the unique family of inflammatory cytokines, produced exclusively by newly defined Th17 cell subset. Particularly important for the role of IL-17 in host defense is its ability to link T-cell function and hematopoiesis through stimulation of granulopoiesis and neutrophil trafficking. However, as demonstrated in our previous studies, IL-17 also affects the cells of erythroid lineage stimulating development of early erythroid progenitors, BFU-E, but suppressing the growth of late stage erythroid progenitors, CFU-E, from normal murine bone marrow. *Aims.* Although we also revealed that at least part of *in vitro* IL-17 effect related to the inhibition of CFU-E is mediated by p38 MAPK phosphorylation, the mechanisms and signaling cascades underlying erythopoietic effects of IL-17 remained largely unknown. The aim of the present study was to investigate the involvement of other two members of MAPK family, JNK and ERK, as well as GATA hematopoietic transcriptional regulators, in IL-17-mediated effects on murine bone marrow erythropoietic progenitors. *Methods.* The contribution of JNK and ERK1/2 MAPKs to the effects of IL-17 on proliferation and differentiation of erythropoietic progenitors was examined by culturing murine bone marrow cells in methylcellulose with IL-17, in the presence or absence of specific pharmacological inhibitors of JNK and MEK1/2-ERK1/2 MAPK pathways. In addition, the influence of IL-17 on the expression of GATA-1, which is essential for terminal erythroid differentiation, and GATA-2, transcription factor involved in expanding primitive hematopoietic progenitors, was determined in murine bone marrow cells by immunoblotting. *Results.* The obtained results demonstrating that pharmacological inhibitor of the MEK1/2-ERK1/2, PD98059, abrogated the growth inhibitory effect of IL-17 on CFU-E, indicated the role of MEK/ERK MAPK pathway in IL-17-induced inhibition of CFU-E growth. Besides, for the IL-17-induced stimulation of BFU-E progenitor cells, the involvement of JNK and/or MEK/ERK MAPK pathways was suggested, since pharmacological inhibitors of both JNK and MEK1/2-ERK1/2 MAPKs, SP600125 and PD98059 respectively, decreased the growth stimulatory effect of IL-17 on BFU-E. Furthermore, enhanced expression level of GATA-1 and no effect on GATA-2 was observed in murine bone marrow cells after IL-17 stimulation. Also, no evidence for JNK and ERK MAPK signaling in IL-17-mediated effects on GATA-1 was demonstrated, since in both unstimulated and IL-17-stimulated bone marrow cells, JNK and MEK1/2-ERK1/2 inhibitors enhanced GATA-1 expression, implicating the role for these MAPKs in GATA-1 downregulation. *Conclusions.* These observations pointed to the involvement of JNK and ERK MAPKs in IL-17-induced effects on both early and late stage erythroid progenitors, BFU-E and CFU-E, from murine bone marrow. Moreover, the results concerning transcription factors evidenced a role for GATA-1 in erythropoietic activity of IL-17, which, in light of previous data demonstrating that overexpression of GATA-1 inhibits differentiation of erythroid cells, could be related to IL-17-mediated inhibition of CFU-E growth.

Chronic lymphocytic leukemia - Biology 1

0093

CPG OLIGONUCLEOTIDE AND INTERLEUKIN-2 STIMULATION REVEAL UNEXPECTED PROGNOSTICALLY RELEVANT CHROMOSOMAL LESIONS IN B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA (B-CLL)

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Current evidence suggests that the CpG oligonucleotide (ODN) in combination with interleukin-2 (IL-2) (ODN+IL-2) can increase CC detection rate of chromosomal abnormalities and complement FISH results. Based on these findings, the present study analysed the results obtained by the ODN+IL2 combination, pokeweed mitogen (PKWM) and iFISH in seventy-two B-CLL diagnosed at our Institution between January 2007 and December 2009. Moreover, it evaluated the existence of any possible correlations between chromosomal findings and clinical outcome. There were forty-five males and twenty-seven females with a median age of 62 years (range 42-83). According to Binet, forty-one patients (56.1%) were considered as stage A, twenty-four (32.8%) as stage B and eight (10.9%) as stage C. PKWM revealed clonal abnormalities in nine patients (12.3%) and did not yield any mitotic figure in eight patients (10.9%), whereas the ODN+IL-2 combination revealed clonal defects in 59% of patients and did not provide any mitotic figures in only three patients (4.1%). In five patients both cultures demonstrated the same clonal defect in a similar percentage of metaphases. In addition, the ODN+IL-2 combination revealed a complex karyotype (≥ three defects) in eight patients (10.9%), two chromosomal defects in eight patients (10.9%) (including four with the rearrangement of band 17p13 and two with the rearrangement of band 11q13), a +12 in ten patients (13.6%) and various translocations in eleven patients (15.0%). iFISH was carried out with the B-CLL FISH probe panel (Vysis, Downers Grove, IL, USA) and revealed clonal abnormalities in 67.1% of patients. The most common defects were: 13q- (46.5% of patients), +12 (10.9% of patients), 11q- (6.8% of patients) and 17p- (5.4% of patients). Two patients with a normal FISH pattern presented a +12 in ODN+IL-2 cell cultures and all the four with the loss of one p53 signal on iFISH showed structural defects of band 17p13 with the ODN+IL2 combination. In addition, when the thirty-four patients with a 13q- on iFISH were investigated with the ODN+IL2 combination, fourteen (41.1%) presented a normal chromosomal pattern, eight (23.5%) another defect, six (17.6%) a complex karyotype, three (8.8%) a 13q deletion and three no *Results.* From a clinical point of view, all the eight patients who showed a complex karyotype were classified as stage C; among the eleven patients who harboured chromosomal translocations, seven were classified as stage B and four as stage A; all the other patients were classified as stage A. In conclusion, i) the results provided by the ODN+IL2 combination and iFISH are complementary since the former examines the entire karyotype and the second specific chromosomal regions; ii) due to their sub-microscopic nature, 13q- and 11q- were rarely revealed by the ODN+IL-2 combination which may probably stimulate a minor +12 clonal cell population as confirmed by the lack of trisomic nuclei on iFISH; iii) the loss of one p53 signal on iFISH is frequently due to an unbalanced rearrangement; iv) complex karyotypes and chromosomal translocations are associated with an advanced Binet stage and 13q deletions if included in a complex karyotype lose their favourable prognostic impact.

0094

INCIDENCE OF CYTOGENETIC ABNORMALITIES IN NEWLY DIAGNOSED BINET STAGE A B-CLL AND RELATIONSHIP WITH PROGNOSTIC BIOMARKERS: UPDATED RESULTS ON 319 PATIENTS INCLUDED IN THE PROSPECTIVE O-CLL1 GISL STUDY

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Background. Biologic risk factors such as immunoglobulin variable heavy chain (IgVH) gene mutation status and CD38 and ZAP-70 expression levels, along with genomic aberrations, have been identified in B-CLL and their prognostic impact has been intensively evaluated in the disease. *Aims.* We investigated the incidence of the known major cytogenetic alterations (+12 and 13q14, 17p13, 11q23 deletions) in Binet A B-CLL patients included in the prospective multicenter O-CLL1 GISL trial. The study was performed by FISH in 319 out of 377 patients enrolled to date. *Methods.* Molecular markers characterization and FISH analyses were previously reported (Cutrona et al. Haematologica, 2008; Fabris et al. GCC, 2008). *Results.* At least one abnormality was found in 209/319 (65.5%) cases. The most frequent abnormality was del(13)(q14), which was detected in 160 cases (50%) followed by +12 (42/319, 13 2%) (one case harboring 17p13 deletion), del(17)(p13) (8/319, 2.5%) and del(11)(q23) (18/319, 5.6%). 13q14 deletion was found as a sole abnormality in 142 (44.5%) patients; in the remaining cases, it was combined with +12 (3 pts) and 17p13 (4 pts) or 11q23 deletions (11 pts). The 13q deletion was found as a monoallelic deletion in 127/160 (79.3%); in the remaining 33 cases the presence of a biallelic deletion was found in >20% of interphase nuclei. No acquisition of new cytogenetic aberrations was evidenced among the 13 patients developing progressive disease (range, 6 to32 months; median, 20 months). In only one case, the proportion of nuclei with 17p13 and 13q14 deletions increased from the time of diagnosis (from 33% to 92%). Biomarkers data were available in all of the patients. CD38 percentages (mean value±sem) were 9.3±1.5, 16.2±2.0, 53.4±5.4, 23.3±1.1,47.3±13.6, 35.1±10.3 for del(13)(q14), normal karyotype, +12, del(11)(q23), del(17)(p13) and multiple alterations, respectively (P<0.0001). The percentages of IgVH mutations significantly correlated with cytogenetic alterations; namely, 5.5±0.3 for cases with del(13)(q14), 4.6±0.4 for normal karyotype, 2.6±0.5 for +12, 0.3±0.2 for del(11)(q23), 1.9±1.1 for del(17)(p13) and 1.2±0.6 for multiple alterations

(P<0.0001). Similarly, a significant correlation was found for ZAP-70 expression: namely 33.5±1.8 for cases with del(13)(q14), 38.6±2.2 for normal karyotype, 47.5±3.5 for +12, 71.4±8.2 for del(11)(q22), 38.3±12.5 for del(17)(p13) and 46.0±6.1 multiple alterations (P<0.0001). Finally, cytogenetic abnormalities were clustered in 3 risk groups [i.e. low del(13)(q14) and normal; intermediate (+12); and high risk del(11)(q23) and del(17)(p13)] and correlated with a scoring system in which patients were stratified in 4 different groups according to the absence (group 0) or presence of 1 (group 1), 2 (group 2) or 3 (group 3) biomarkers (Morabito et al., BJH, 2009,). Notably, 154/162 cases scoring 0, gathered in the low FISH group, whereas 16/20 high FISH risk cases clustered in scoring 2-3 (P<0.0001). *Conclusions.* Our data indicate that cytogenetic abnormalities predicting unfavorable prognosis show a relatively low incidence in newly diagnosed Binet stage A B-CLL patients and are significantly associated with negative prognostic biomarkers predictive of disease progression. Furthermore, preliminary results in a limited number of cases indicate that the acquisition of new abnormalities seem to be an infrequent event during disease progression.

0095

TP53 MUTATIONS IN CHRONIC LYMPHOCYTIC LEUKEMIA: ANALYSIS OF 2,435 CONSECUTIVE CLL SAMPLES

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Background. TP53 is a transcription factor that plays pivotal role in the process of DNA repair and apoptosis. In 10-20% of patients with chronic lymphocytic leukemia (CLL) the TP53 pathway is affected by genomic TP53 mutation, aberrant mRNA splicing or by dysfunction of cooperating protein partners. It has been reported that in CLL, chemotherapy induces evolution of TP53 mutated subclones that might lead to the progression of the disease. In our study (from January 2005 to December 2009), we have analyzed TP53 mutation status in 2,435 consecutive CLL samples, including 1,287 diagnostic samples and 1,148 samples during the follow-up. The follow-up comprised 181 CLL patients, for whom clone-specific assays to monitor minimal residual disease have been prepared. *Aims.* The aim of the study was to address mutational status of TP53 gene, modes of inactivation/modulation of the TP53 gene and the possibility of clonal evolution of TP53 mutated subclones in a large cohort of 2,435 CLL patients. *Methods.* At the time of diagnosis, mutational status of TP53 gene was determined using direct sequencing and functional assay FASAY, as described elsewhere.[1] Assays to monitor minimal residual disease (MRD) have been prepared based on the sequence of clonal IgVH rearrangement(s) identified at the time of diagnosis, as described in.[2] Nine temperature-sensitive mutants TP53 and a novel splicing variant delta ex6 were molecularly cloned and characterized.[3] *Results.* In a cohort of 1,287 diagnostic CLL samples, we have identified 237 TP53 variants (18.4%), including point mutations, insertions, deletions, temperature-sensitive mutations and aberrant splicing variants (Table 1).

Table 1 shows the list of TP53 mutants, identified in our cohort of 1,287 diagnostic CLL samples, which in vitro biological properties have been tested using functional assay FASAY. Temperature-sensitive mutants are highlighted in blue.

Mutation	Nr.	Mutation	Nr.	Mutation	Nr.	Mutation	Nr.	Mutation	Nr.	Mutation	Nr.
R6X	3	K164E	1	H214R	1	P250L	1	D281G	1	delta ex6	6
W91X	1	G167X	1	Y215I	1	P250S	1	D281V	2	delta ex6 + beta	11
K101E	1	V172A	1	V216M	1	Y251V	1	P252W	1	beta	10
P109I	1	V173A	2	P219H	1	Y255T	2	R282C	7	ins AGT at nt 780	1
R110L	2	R175H	2	Y220C	4	T256P	1	T284S	1	ins G at the border ex5/in5	1
R111S	2	C176R	2	Y220H	1	R258G	1	R284G	1	ins TAT at the border ex7/in8	1
Y126C	2	C176W	1	F222L	1	P258K	1	K305M	1		
Y126M	1	P177L	1	S227P	1	D259G	2	R320E	1		
K132N	2	M178R	1	W231R	4	D259S	1	R320T	1		
Q136E	1	P170S	1	Y234C	5	R266R	1	A336P	1		
A138V	1	H179L	1	Y234D	3	R267P	1	delint 302	1		
K139R	2	H179R	4	Y234H	1	R267W	1	delint 320-329	1		
C141Y	1	P181S	1	M236R	2	R273C	2	delint 603-678	1		
Q144X	2	D184N	1	M237I	2	R273H	4	delint 504-678	1		
W146X	1	G187D	1	M239D	1	P27JG	1	delint 535-559	1		
P151S	1	L188P	1	L241C	1	V274A	4	delint 550-576	1		
P152L	1	P190H	1	M243T	1	C275G	1	delint 636	1		
P153L	1	P190S	1	G245D	1	C275Y	1	delint 704-709	1		
R156P	1	H193L	2	G245S	8	A276V	1	delint 716-736	1		
V157F	2	H193R	2	R247D	1	C277F	1	delint 724-739	1		
V157G	1	L194P	1	P248Q	1	C277Y	2	delint 749-751	1		
A159P	2	P196R	1	P248W	8	R280K	1	delint 792-794	1		
A161T	3	T205C	1	P249S	3	R280P	1	delint 828	1		
Y163H	2	P212R	1	P249W	1	R280T	1	delint 522-549	1		

227 TP53 variants have fully penetrant transactivation-defective mutant phenotype *in vitro*. Additional 10 mutants display temperature-sensitive properties with mutant phenotype at permissive conditions and wild type phenotype at non-permissive conditions. In 1,148 follow-up samples analyzed for the MRD level in 181 CLL patients undergoing chemo/immunotherapy or bone marrow transplant we have never observed clonal evolution, contrary to the data described in.[4] *Summary/Conclusions.* TP53 mutations/variants are diverse and affect most of the coding sequence of TP53. Though there are few known hot-spot mutations with proven biological functions, there is still a large number of TP53 mutations, which biological functions have not been assessed previously. The majority of TP53 mutations/variants are activating, but some of them have unusual characteristics (e.g. temperature sensitivity), or might represent aberrant splicing isoforms expressed at variable levels over the time. Moreover, results based on 1,148 follow-up CLL samples indicate that evolution of mutated TP53 subclones triggered by chemotherapy does not seem to play a major role in CLL progression.

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0096

TP53 GENE SEQUENCING IDENTIFIES A PECULIAR MUTATION PROFILE AND CORRELATION WITH P53 PROTEIN DYSFUNCTIONS IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS AT DIFFERENT PHASES OF THE DISEASE

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Background. Several biological and genetic properties in chronic lymphocytic leukemia (CLL) patients have an important prognostic value for the stratification into risk categories. TP53 mutations, as well as 17p deletions, make the disease particularly aggressive and chemoresistant. *Aims.* To evaluate the incidence and profile of TP53 mutations in CLL patients at different phases of the disease and assess their correlations with specific p53 functional alterations. *Methods.* We investigated 446 CLL patients: 158 at diagnosis, 234 at progression and 54 with chemoresistant disease. Screening for TP53 mutations (exons 4-9) was performed by sequencing. p53 function was examined by immunoblotting on CLL cells, after exposure to ionizing radiation (IR). IR-induced apoptosis was verified using annexin/propidium iodide staining and flow cytometry analysis. *Results.* We found an overall incidence of TP53 mutations of 8% (36/446 patients) with the subsequent distribution: 4/158 (2.5%) patients at diagnosis, 23/234 (9.8%) patients with progressive disease and 9/54 (16.7%) chemoresistant patients. In all groups, TP53 mutations were mainly missense mutations in exons 5-8, with the highest incidence in exon 8, but we also observed 4 nonsense mutations and 4 microdeletions exclusively affecting progressive and chemoresistant patients. Among 37 mutations, 25 (67.6%) showed a heterozygous and 12 (32.4%) a homozygous status. In particular, 25/33 (75.7%) missense mutations were transitions and 8 of 33 (24.3%) were transversions. Eighty-two CLL patients were examined for p53 functionality. Sixty patients had a normal p53 response to IR. Apoptosis was significantly (P=0.07) higher after IR (53.2%±15.2 vs 74.6%±14.1). All these patients showed a wild type TP53 gene sequence. Twenty-two patients, 1/12 at diagnosis, 12/51 with progressive and 9/19 with chemoresistant CLL, showed an impaired p53 response to IR. In 12/22 (54.5%) p53 was present and increased after IR (type I dysfunction); in 9/22 (41%) p53 was present but did not increase after IR (type II dysfunction) and in 1/22 (4.5%) p53 was undetectable also after IR (type III dysfunction). TP53 mutations were detected in all dysfunctional samples except for 5 chemoresistant CLL. In these latter cases, other potential causes of p53 dysfunction are under investigation. In 7/9 type I dysfunction was associated with heterozygous missense TP53 mutations in the DNA binding domain (exons 5-8). Six of 7 patients with type II dysfunction showed missense mutations but mainly in homozygous status. Furthermore, type II dysfunction was associated with TP53 mutations affecting not only exons 6 and 8 but also exons 4 and 9; no mutations within exon 7 were observed. Finally, type III response was associated with a nonsense homozygous mutation that introduced a

stop codon at position 213 of exon 6. Interestingly, type I dysfunctions were partially resistant to radiation-induced killing (40.4±13.9% pre- vs 50.5±16.9% post-IR), while type II and III dysfunctions were completely radioresistant (63.4±28.2% pre- vs 62.9±28.3% post-IR). *Conclusions.* We showed the highest incidence, as well as the more complex TP53 mutations and dysfunctions, among progressive and chemoresistant CLL patients. The association between p53 dysfunctions and TP53 mutations, observed in this study, may help to improve the characterization of TP53 mutated patients.

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TP53 MUTATIONS ARE INFREQUENT IN NEWLY-DIAGNOSED CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Chronic lymphocytic leukemia (CLL) patients frequently carry TP53 mutations in conjunction with deletion of the remaining 17p allele. However, recent studies have reported that a small proportion (3-5%) of CLL patients bearing TP53 mutations can display an intact 17p allele. These patients tend to have rapid disease progression, display a complex karyotype and are associated with chemorefractoriness. *Aim.* While *TP53* mutation frequencies have mostly been reported on referral cohorts, which may therefore have a biased collection of more aggressive cases, the aim of the present study was to determine the *TP53* mutation frequency in a newly-diagnosed cohort of CLL patients from a population-based material. *Methods.* A total of 268 Scandinavian CLL patients were analyzed for recurrent genomic aberrations and mutation of the *TP53* gene by sequencing of exons 4-8. *Results.* Representing the indolent nature of this cohort, 67% of patients harbored mutated IGHV genes, 77% were in Binet Stage A and 76% had either no recurrent aberration or displayed 13q-deletions. 17p-deletion was detected in only 10 cases (3.7%). Overall, *TP53* mutations were detected in 10 of 268 (3.7%) CLL patients; where 7 cases showed a concomitant 17p deletion and only 3 (1.1%) carried *TP53* mutations without 17p-deletion. Furthermore, we confirmed a significant decrease in overall survival (P<0.0001) and time to treatment (P=0.01) for patients with both *TP53* mutations and 17p-deletions, compared to patients without any mutation or deletion. Of three patients with only *TP53* mutations, all displayed mutated IGHV genes. One patient displayed a trisomy 12 and died 66 months after diagnosis while the remaining two patients displayed 13q-deletions and were alive >9 years after diagnosis. This is in line with the recent notion that 17p-deleted patients with mutated IGHV genes can display a more stable disease. *Summary.* We confirm the high prevalence of *TP53* mutation in 17p-deleted patients, and the associated poor survival of this patient group. The prevalence of *TP53* mutations in the absence of 17p-deletion appears lower in newly diagnosed CLL patients than the frequency reported by earlier studies. Thus, this finding further supports the notion that *TP53* mutations are rare at disease onset and instead arise during CLL progression. FM, NZ and MK contributed equally as first authors.

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CLL-SPECIFIC RESEQUENCING MICROARRAY AS A TOOL FOR CLL RESEARCH AND DIAGNOSTICS

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Background. Chronic lymphocytic leukemia (CLL) is characterized by a progressive accumulation of functionally incompetent B lymphocytes. Its highly variable clinical course is mostly determined by the combination of two biological factors: mutational status of the immunoglobulin heavy-chain variable region (IgVH) and four prominent genomic aberrations, i.e. deletions 11q22-23, 13q14, 17p13 and trizomy of chromosome 12. An impact of gene mutations on CLL progression is much less clear. Recently, several studies have shown a clear association between mutations in the TP53 (locus 17p13) or ATM (locus 11q22-23) tumor-suppressor genes and disease progression and chemo refractoriness. *Aims.* The aim of this project was to introduce a fast and reliable tool for resequencing of the selected genes, whose alterations may have a negative impact on CLL pathogenesis and/or therapeutic response. *Methods.* We have designed a custom resequencing microarray (Affymetrix, 50K) for exons and exon/intron splicing sites for the genes TP53, ATM, BCL6, RB1, CDKN2A, C-MYC, FAS, SH2D1A (SH2 domain-containing protein 1A), SOCS1 (suppressor of cytokine signaling 1) and for 110 pre-micro RNAs including approx. 20 nucleotides from pri-miRNAs. The selected genes were amplified from genomic DNA using long range PCRs ranging from 1 (e.g. C-MYC) to 19 (ATM) reactions per gene. Amplicons were pooled, fragmented and cohybridized on the array according to the manufacturer's protocol. *Results.* In this ongoing project we focus primarily on the high-risk CLL patients. Until now, 25 samples including 4 wt/healthy controls and 21 CLL patients have been sequenced. 11 harbored TP53 mutation previously identified by using different methodology (functional yeast analysis coupled with direct sequencing) and 10 manifested ATM deletion in >90% of cells. Known mutations in the TP53 gene served for assessing of the microarray ability to identify various types of mutations. We have observed that all missense mutations and common polymorphisms are readily identifiable. Short (1-2 nt) and long deletions (tens of nt) are detectable only if the second allele is missing (detected by FISH). Until now, we have detected four mutations and six common SNPs in the ATM gene; in all cases the mutations accompanied 11q22-23 deletion on the second allele. Two of them were missense substitutions, which had been detected previously in lymphoid tumors; remaining two affected splicing sites of the exons 28 and 46. So far, eight SNPs have been detected by the resequencing analysis in the miRNAs; one in the mature miRNA (miR-412), four in the pre-miRNAs (pre-miR-453, pre-miR-146a, two in pre-miR-27a) and three in the pri-miRNAs (pri-miR-154, pri-miR-100, pri-miR-126). *Summary/Conclusions.* The resequencing microarray may serve as an appropriate tool to dissect some important questions concerning the high-risk CLL. It can bring insight into e.g. mutual relationship between inactivation of the TP53 and ATM genes, identification of mutations in the miRNAs related to CLL pathogenesis or detection of potential mutations in the RB1 gene in cases with 13q14 deletion encompassing the corresponding locus. In comparison with classical sequencing approaches, the resequencing methodology is more robust, cheaper and much less time consuming.

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EVALUATION OF TP53 MUTATIONS WITH THE AMPLICHIP P53 ARRAY IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL): HIGHER SENSITIVITY AND CORRELATION WITH CLINICAL OUTCOME

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Background. Given the growing evidence that TP53 alterations are predictors of progression and survival in chronic lymphocytic leukemia (CLL), its screening is essential in an optimal patients' management. *Aims.* We evaluated 98 untreated CLL cases for TP53 mutations combining direct sequencing and the AmpliChip p53 Research Test, a microarray-based resequencing assay (Roche Molecular Systems, Inc., Pleasanton, CA). The results obtained were compared and correlated with different clinico-biological parameters. Finally, we studied the gene expression profile associated with TP53 alterations. *Methods.* For sequencing analysis, TP53 exons 5, 6, 7 and 8 were amplified from genomic DNA by PCR and the expected products were excised from the gel and purified. Sequences data were compared with wild-type (WT) sequences. The AmpliChip p53 Research Test allows sequencing of exons 2-11: genomic DNA was amplified in two separate PCR reactions. Subsequently, PCR products were fragmented, hybridized, stained and scanned. Each array queries 1268 nucleotide positions, investigated by individual probesets that contain five probes: 1 for WT sequence, 3 for possible single base substitutions and 1 for single base deletions. Each probe contains multiple copies of an oligonucleotide sequence. The mutation detection algorithm detects single base pair substitutions and deletions. Samples from CLL patients also underwent microarray analysis using the HGU133 Plus 2.0 Affymetrix arrays. T-test analysis was

performed comparing TP53-mutated vs WT samples and del(17p) cases vs the remaining CLL, respectively. *Results.* The AmpliChip p53 Research Test detected 17 mutations (17.3%) in 14 patients, with an incidence of 17.2% vs 7.1% in progressive vs stable cases, respectively; a significant association between TP53 mutations and del(17p) was recorded. Comparison with TP53 sequencing indicated a higher sensitivity of the AmpliChip p53 Research Test: 8 mutations were detected by both techniques, 9 only by the array, although 3 were in exons not routinely screened by sequencing, while the latter failed to identify 2 microdeletions.TP53 mutations, detected by the AmpliChip p53 Research Test, were associated with a worse Overall Survival (P=0.0002). Furthermore, we identified a common pattern of gene expression in CLL cases with TP53 alterations, with a more distinct signature associated with del(17p) rather than TP53 mutations. Finally, a high percentage of codon 72 polymorphism was recorded in CLL patients. *Conclusions.* The AmpliChip p53 Research Test is a simple and non-time consuming method, that appears to be more sensitive than sequencing, and useful as a prognostic tool. This study confirms a high percentage of TP53 mutations in CLL with unfavorable outcome and a significant association between TP53 aberrations and del(17p). TP53 alterations were coupled with a specific gene profile, with a more distinctive signature associated with del(17p) than TP53 mutations, most likely due to a concomitant gene dosage effect. Further investigations on the role of codon 72 polymorphism, highly represented in CLL, are ongoing.

0100

TRANSLOCATION IS A FREQUENT MECHANISM FOR 17P DELETION IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Deletion of TP 53 gene located on 17p13.1 are detected by interphase fluorescence *in situ* hybridization (FISH) in about 8-10% of chronic lymphocytic leukemia (CLL) patients. This deletion is associated with an aggressive disease and poor therapy response. Recent studies using conventional metaphase cytogenetics in some cases revealed that mechanism other than interstitial deletion could lead to loss of critical chromosomal regions in CLL cells. *Aims.* The aim of our study was to characterize cytogenetic mechanism of 17p deletion in CLL patients using advanced methods of metaphase cytogenetics. *Methods.* We performed metaphase cytogenetics, metaphase FISH and multicolour FISH (M-FISH) analysis on cohort of 27 CLL patients with TP 35 deletion previously detected using interphase FISH. Metaphase analysis was performed on peripheral blood samples using 72 hour cultivation with CpG oligonucleotides and IL-2 stimulation. Metaphase FISH with various DNA probes (Abbott-Vysis, Downers Grove, IL, USA) and multicolour FISH with 24-color painting probe mix (MetaSystems, Altusshein, Germany) were also performed. *Results.* Metaphase cytogenetics, FISH and M-FISH analysis of 27 patients with TP 53 deletion revealed: 8 patients with a simple deletion of short arm of chromosome 17, one patient with 17p deletion caused by isochromosome 17q formation and 18 patients (67%) with 17p affected by unbalanced translocation. Unbalanced translocation associated with formation of dicentric chromosome on derivative chromosome 17 was detected in 11 patients and pseudodicentric chromosome was present in 2 patients. Four patients had a dic(17;18)(p11.2;p11.2); in other 7 cases with dicentric formation, the chromosome partners were as follows: 4p, 8q, 9p(2x), 20p, and 21p(2x). In 5 patients with 17p translocation without dicentric chromosome, 1p/13q, 3q, 4p, 12q and 15q were translocated on 17p. In two cases, 2 resp. 4 independent clones with 17p unbalanced translocation with different partner chromosomes were detected. Unbalanced translocation cases were associated with del 13q (5x), del 11q (1x) and trisomy 12 (1x), detected by interphase FISH; cases with 17p deletion had only del 13q (5x). In cases with 17p translocation, cytogenetic analysis shown that substantial part or complete short arm is probably missing due to translocation, but this fact should be proven by sequential metaphase FISH analysis using different 17p probes. In total, 13 out of 18 patients with translocation and 5 out of 8 patients with deletion had complex chromosomal aberrations. There was no difference in number of patients with comlex karyotype and mean number of aberrations per case between 17p deletion and 17p translocation groups. All 14 patients with 17p translocation and 5 of 6 patients with 17p deletion with available data had unmutated IgVH. Correlation with selected clinical and biological data of patients will be presented. Summary. We confirmed that unbalanced translocation is a frequent mechanism for 17p deletion in CLL patients. 17p deletion due to unbalanced translocation is afrequently accompanied by dicentric chromosome formation. Dic(17;18)(p11.2;p11.2) seems to be a recurrent chromosomal aberration in CLL.

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0101

GENE EXPRESSION SIGNATURE OF P53 MUTATED AND/OR DELETED CHRONIC LYMPHOCYTIC LEUKAEMIA CELLS IDENTIFIES ARHGDIA AS A GENE UNIVERSALLY OVEREXPRESSED IN CLL

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Background. p53 deletion at 17p13.1 and p53 mutations have been identified as having adverse prognostic relevance in B-cell chronic lymphocytic leukemia (CLL). In fact, disruption of p53 by point mutations and/or deletion at 17p13 occurs in a fraction of cases at diagnosis and predicts poor survival and chemorefractoriness. *Aims.* to analyze the gene expression profile (GEP) related to p53 "disruption" in CLL. *Methods.* purified cells from twenty PB CLL samples, characterized for p53 mutations and including 13 p53 wild type ($p53^{wt}$) cases, 7 p53 deleted or mutated ($p53^{del/mut}$) cases of which 5 with del17p13 and p53 mutations, 1 with del17p13 alone, and 1 with p53 mutations alone, were utilized for performing gene expression profile (GEP) experiments; bioinformatic analyses of GEP data were performed with gene set enrichment analyses (GSEA); validations and quantitative gene expression studies on CLL and normal B cells were performed by quantitative real-time PCR (qRT-PCR). *Results.* comparing the constitutive GEP of 13 $p53^{wt}$ versus 7 $p53^{del/mut}$ cases, we obtained 72 (26 up-regulated, 46 down-regulated of which 31 belonging to the 17p segment) differentially expressed genes in $p53^{del/mut}$ cases. The transcripts representing the constitutive gene expression signature of $p53^{del/mut}$ CLL cases were tested with GSEA, focusing on the positional gene sets corresponding to human chromosome and cytogenetic bands. According to this analysis, the gene set containing genes related with the short arm of chromosome 17 presented the lowest nominal P (P=0.002, FDR-q=0.001). Consistently, $p53^{del/mut}$ CLL cases were characterized by a significant enrichment in down-regulated genes located in the 17p segment, including several genes known to display proapoptotic activities (e.g. *PSMB6*, *RPL26* and *ZBTB4*) whose lower expression in $p53^{del/mut}$ compared to $p53^{wt}$ cases was confirmed by qRT-PCR validations (PSMB6, P=0.003; RPL26, P=0.03; ZBTB4, P=0.02). Notably, 26 out of 72 differentially expressed genes turned out to be upregulated in $p53^{del/mut}$ CLL. Among these genes we focused on *ARHGDIA*, a Rho GDP dissociation inhibitor with a well-known antiapoptotic activity mediating cellular resistance to chemotherapy agents. The up-regulation of this gene in $p53^{del/mut}$ versus $p53^{wt}$ CLL cases was confirmed in qRT-PCR experiments (P=0.0257). Moreover, using the same qRT-PCR approach, overall higher expression levels of *ARHGDIA* were detected in a different series of 43 CLL samples when compared to normal B cells of PB samples from 15 healthy donors (P<0.0001). Furthermore, no differences in *ARHGDIA* expression levels were detected between various CLL categories, obtained according to IGHV gene status, FISH groups, and CD38, CD49d or ZAP-70 expression, although CLL cases carrying a 17p13 deletion (6 cases) were among those expressing high levels of *ARHGDIA* transcripts. *Conclusions.* the gene expression signature of $p53^{del/mut}$ CLL cases is in keeping with the higher aggressiveness and chemorefractoriness of $p53^{del/mut}$ CLL. Moreover, in agreement with *ARHGDIA* overexpression detected in other human tumors (e.g. ovarian, breast and colorectal cancers), the higher expression levels of *ARHGDIA* in CLL compared to expression levels of normal B cells from healthy donors points out a possible role for *ARHGDIA* as new therapeutic target or early detection biomarker.

0102

ARRAY-BASED GENOMIC PROFILING OF CHRONIC LYMPHOCYTIC LEUKEMIA AT DIAGNOSIS AND FOLLOW-UP

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Background. Although no common genetic defect has been described in chronic lymphocytic leukemia (CLL), recurrent genomic aberrations (i.e. deletions of chromosome 11q, 13q, 17p and trisomy 12) are important for prognostication. Recent development of high-resolution microarray techniques provides effective detection of the known recurrent aberrations and simultaneous exploration of overall genetic complexity such as small copy-number alterations (CNAs) and copy-number neutral loss of heterozygosity (CNN-LOH), which may contribute to pathogenesis and prognosis in CLL. In addition, comparison of array based genomic profiles from CLL samples taken at diagnosis and at follow-up allows a detailed evaluation of clonal evolution (CE). *Aims.* To investigate genomic aberrations in newly diagnosed CLL patients by applying high resolution SNP-arrays, and to evaluate CE in follow-up samples from patients grouped according to treatment status and IGHV mutation status. *Methods.* 250K SNP-arrays were applied for identification of CNAs and CNN-LOH in 370 CLL samples taken at diagnosis from a population-based Scandinavian cohort and in 43 follow-up samples obtained 5-8 years from diagnosis. *Results.* Genomic aberrations were identified in 90% of diagnostic samples, where the majority of samples (71%) carried between 1 and 3 CNAs, 16% showed 4-9 aberrations, while only 2% were highly complex (≥10 CNAs). The known recurrent alterations were found in 70% of patients; del(13q) 55%, trisomy 12 10.5%, del(11q) 10%, and del(17p) 4%. The deletions of 13q covered the miR-15/miR-16 in 96% of cases and the ATM and TP53 genes where included in all cases with 11q and 17p deletions, respectively. Additional recurrent CNAs included gains of chromosome 2p (2.2%), 8q (1.9%) and deletions of 4p (1.4%), 8p (1.9%) and 14q (1.6%). Genomic complexity was correlated to an unfavorable outcome, however, the patients with a higher number of CNAs most often carried the poor prognostic del(11q) and del(17p). Recurrent copy-number neutral loss-of-heterozygosity (CNN-LOH) was detected on chromosome 13q in 13 samples (3.5%) and 11 of these patients also carried a homozygous del(13q). Interestingly, in the follow-up study, CE was observed in 8/18 (44%) patients with unmutated IGHV genes, and in 4/15 (27%) IGHV mutated and treated patients. In contrast, untreated IGHV mutated patients (n=10) did not acquire additional aberrations. Acquisition of del(13q) was the most common secondary event, detected in both IGHV unmutated and mutated patients, whereas aberrations on chromosome 6q, 8p, 9p and 10q were only acquired in IGHV unmutated patients. *Summary*. Whole-genome screening with SNP-arrays revealed that a high frequency of the newly diagnosed CLL patients carry genomic aberrations, where an increasing genomic complexity was associated with an unfavorable prognosis, but also with the presence of poor-prognostic recurrent aberrations. Evaluation of CNN-LOH identified recurrent regions on chromosome 13q. The presence of a homozygous del(13q) in most of these cases implies a novel mechanism for homozygous loss of miR-15/16 in CLL. Finally, in the follow-up study, CE was associated with aggressive disease, since novel aberrations arose in IGHV unmutated and IGHV mutated/treated patients, but not in IGHV mutated/untreated patients.

0103

HIGH-DENSITY SCREENING REVEALS A DIFFERENT SPECTRUM OF GENOMIC ABERRATIONS IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS WITH 'STEREOTYPED' IGHV3-21 AND IGHV4-34 B CELL RECEPTORS

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Background. Multiple chronic lymphocytic leukaemia (CLL) subsets with virtually identical, 'stereotyped' B-cell receptors (BCRs) have been identified in up to 30% of patients. These subsets show similar immunoglobulin heavy (IGH) and light-chain gene usage, and share an aminoacid identity ≥60% in the heavy complementarity determining region 3 (CDR3), the main determinant of antigen specificity. These striking findings of stereotypy have implied antigen involvement in leukemogenesis. Interestingly, stereotypy has also been indicated to influence the clinical course, e.g. stereotyped IGHV4-34 (subset #4) shows a more indolent disease and a low median age at diagnosis than non-stereotyped IGHV4-34 patients. Conversely, IGHV3-21 patients with stereotyped (subset #2) and non-stereotyped (non-subset #2) BCRs share an equally poor overall survival, independent of IGHV mutational status, although stereotypy has been associated with shorter time to progression and poor-prognostic markers. Today limited knowledge exists about the spectrum of aberrations in these different subsets. *Aim.* Screening for whole-genome events in biologically/clinically divergent CLL with stereotyped/non-stereotyped IGHV3-21 and IGHV4-34 BCRs and comparison to our recent array based study on a population-based CLL material. *Methods.* We applied 250K SNP-arrays to study copy-number aberrations (CNAs) and copy-number neutral loss-of-heterozygosity (CNN-LOH) in stereotyped IGHV3-21 (subset #2, n=29), stereotyped IGHV4-34 (subset #4, n=17; subset #16, n=8), non-subset #2 IGHV3-21 (n=13) and non-subset #4/16 IGHV4-34 (n=34) patients. *Results.* Over 90% of subset #2 and non-subset #2 carried CNAs, whereas 75-76% of subset #4 and subset #16 showed CNAs. Subset #2 and non-subset #2 displayed a higher average number of aberrations compared to subset #4. However, compared to our array-based CLL study, subset #2/non-subset #2 cases were no more complex when accounting for all CNAs. del(13q), the only recurrent aberration detected in subset #4 (35%), was substantially more frequent in subset #2 (79%) compared to other CLL studies. Furthermore, del(11q) encompassing the ATM gene was more frequent in subset #2 and non-subset #2 (31% and 23%) relative to subset #4, non-subset #4/16 patients and other CLL studies. These aberrations may be important in IGHV3-21 pathogenesis, particularly as concurrent 11q and 13q deletions were frequent in subset #2 cases. We detected some novel aberrations, however few were overlapping. A concurrent gain on chromosome 2q and a loss at 3p were identified in two subset #2 cases. In IGHV4-34, three recurrent aberrations were observed on chromosome 2q, 7q and 14q but not in any particular subset. Small non-recurring CNN-LOH regions were frequent in all subset- and non-subset groups. Larger (>3 Mbp) overlapping CNN-LOH regions were detected on chromosome 6 in two non-subset #4/16 patients and on chromosome 20 in one subset #2 and non-subset #2 case. Recurrent CNN-LOH was detected on chromosome 13q (5 cases), independent of BCR stereotypy. *Conclusions.* Genomic aberrations were more common in subset #2 and non-subset #2 compared to subset #4. High del(11q) frequency in subset #2 patients may be linked to their adverse outcome. Conversely, the lower prevalence of CNAs and the absence of poor-prognostic aberrations in subset #4 may reflect an inherent low-proliferative disease preventing accumulation of genomic alterations.

0104

CHRONIC LYMPHOCYTIC LEUKEMIA WITH DELETION OF 13Q14 OR 11Q22-23 - EVIDENCE FOR A COMMON PATHOGENETIC PATHWAY FROM GENE EXPRESSION DATA

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Background. Chronic lymphocytic leukemia (CLL) is a heterogeneous disease with survival rates ranging from months to decades. About 40% of CLL cases have deletions of chromosomal band 13q14 and approximately 10% of cases show deletions in chromosome bands 11q22-23. Cases with 13q14 deletion as sole abnormality have a good prognosis, whereas cases with 11q22-23 deletion have one of the worst prognosis. Interestingly, the 13q14 and 11q22-23 regions show ancestral synteny in the zebrafish genome. It is therefore tempting to speculate that there might be a common molecular mechanism linking 11q- and 13q-deleted cases of CLL. *Aims.* We aimed to examine the gene expression profile of conserved genes located in 13q14 and 11q22-23 in CLL cases with deletions in these regions. *Methods.* Microarray-based gene expression profiling and FISH analysis were performed on 151 CLL patients. In addition, we performed quantitative real time PCR for selected genes (*ARHGAP20* and *TP53*) on 116 well defined CLL samples in which more than 66% of the cells were positive for the FISH marker. *Results.* We examined the 13q14 and 11q22-23 regions for differentially regulated genes in subgroups defined by FISH in our microarray dataset. Surprisingly, the expression levels of the Rho GTPase activating protein 20 (*ARHGAP20*) which is located in the commonly deleted region in 11q22-23 showed - counterintuitively - a significantly higher expression in CLL cases with 11q22-23 deletions compared to cases with no detectable genetic lesion or trisomy 12. Additional analysis using quantitative real time PCR confirmed these observations with *ARHGAP20* being significantly higher expressed in cases with 11q deletions and interestingly also in cases with 13q deletions. *TP53* was used as control gene and showed the expected reduction of expression levels in 17p deleted cases due to haploinsufficiency. *Summary/Conclusions.* Despite its location in the deleted region on 11q22-23, *ARHGAP20* is up regulated in 11q-deleted cases suggesting a complicated mechanism of regulation. Very interestingly, *ARHGAP20* was found to be the target of chromosomal translocations in rare cases of CLL. The *ARHGAP20* gene encodes an evolutionary highly conserved protein and is located in a common ancestral region in the zebrafish genome, harboring genes from human chromosomal regions 13q14 and 11q22-23. The fact that *ARHGAP20* is unexpectedly highly expressed in cases with 11q22-23 deletions and in cases with 13q deletions suggests a molecular connection between 11q- and 13q-deleted cases of CLL. Our findings also suggest a prominent role of *ARHGAP20* in the pathogenesis of CLL.

0105

DETAILED ANALYSIS OF DEL(13Q14) BY HIGH-RESOLUTION SNP ARRAY AND PROGNOSIS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Genomic aberrations are important prognostic factors in chronic lymphocytic leukemia (CLL) [Döhner et al., NEJM, Dec. 2000]. Among those del(13q14) is the most frequent one and has been associated with a good prognosis. Its underlying pathogenetic mechanism, however, is still not fully understood. With the development of single-nucleotide polymorphism (SNP) arrays a method has become available for high-resolution genome profiling. We sought to precisely map 13q deletion sizes and correlate the molecular data with clinical outcome. *Material and Methods.* We studied samples of CLL patients enrolled in the CLL8 trial of the German CLL Study Group (GCLLSG). DNA of CD19 separated mononuclear cells was hybridized to the Affymetrix® 6.0 SNP array. Unpaired analysis for DNA copy number changes was performed against 20 own reference samples using the aroma affymetrix software package. The range of each del(13q) was revised by displaying the normalized data in dCHIP SNP. FISH analysis (13q, 11q, 12q and 17p) was performed on all samples. Cases were grouped by their deletion size into quartils and by their copy number status for RB1. Clinical correlation for overall response (OR), overall survival (OS) and progression free survival (PFS) was performed for each group and tested for statistical significance by a Chi-square test. *Results.* Del(13q14) was found in 57% of all samples; in 35,5%, del(13q14) was the sole abnormality as assessed by our routine FISH panel. The minimal deleted region was 277.250 kb in size. On the centromeric site it was defined by the probesets CN_654150 and CN_654151 that have an intermarker distance of 8.692 kb and cover a segment harboring the two microRNAs mir15a and mir16. Deletions on 13q showed a high heterogeneity in size, ranging from 294 kb to 68 Mb; one case had a monosomy 13. Quartile two started with a deletion size of 1.231 Mb, quartile three with a size of 2.687 Mb, and quartile four with a size of 9.057 Mb. Patients with a del(13q) >9 Mb in size (quartile four) had a lower OR rate (P=0.028), a shorter OS (P=0.01) and in trend a shorter PFS (P=0.089). Patients whose deletion encompassed RB1 had in trend a shorter OS (P=0.141), whereas there was no effect on OR or PFS. Among patients who had del(13q14) as sole abnormality, deletion size >9 Mb (P=0.044) and concurrent loss of RB1 (P=0.021) predicted inferior OS, whereas there was no effect on OR or PFS. *Conclusions.* In our patient cohort, the minimal deleted region in 13q14 spanned 277.250 kb and contained the two previously described microRNAs mir15a and mir16. Deletion sizes greater than 9 Mb predicted inferior outcome both in the cohort of all 13q14 deleted patients as well as those harboring 13q14 deletion as sole abnormality, suggesting that additional genes outside the minimal deleted region may add to the pathogenesis of the disease.

0106

INTRACLONAL DIVERSIFICATION OF IMMUNOGLOBULIN GENES IDENTIFIES TWO DISTINCT MOLECULAR SUBTYPES OF RICHTER TRANSFORMATION

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Background. The pathogenetic mechanisms underlying CLL transformation to Richter syndrome (RS) are poorly understood and might involve antigen stimulation. In B-cell neoplasia, analysis of intraclonal diversification (ID) of immunoglobulin (IGHV) genes provides information on tumor immunogenetics. Aim. We investigated ID in RS (all DLBCL), in order to i) define RS clonal evolution; and ii) assess the role of antigen stimulation in clonally related vs unrelated RS. *Methods.* The study was based on 43 samples: i) 10 clonally related CLL/RS pairs; ii) 3 clonally unrelated RS; and, for comparison, iii) 20 *de novo* DLBCL. Fifty IGHV subclones were analysed per sample. Mutations observed in only one subclone were defined unconfirmed (UCM). Partially shared mutations were defined confirmed (CM). The normalized mutation frequency (NMF) was calculated according to the formula: (CM+UCM)/number of subcloned sequences x sequence length. Phylogenetic analyses was performed with MEGA4. In order to define the load of oncogenic molecular lesions, RS were analysed for: i) mutations of TP53, CARD11, TNFAIP3/A20, BLIMP1, BCL6, PIM1, PAX5, TTF, c-MYC, CD79A/B, EZH2; ii) chromosomal abnormalities of BCL2, BCL3, BCL6, c-MYC, MYCN, ATM, TP53, 6q21. Informed consent was obtained. *Results.* Clonally related RS were classified as unmutated (100% homology; 5/10), minimally mutated (99.0-99.9% homology; 2/10) or borderline mutated (98.0-98.9% homology; 3/10). Stereotyped HCDR3 occurred in 5/10 cases (subset 8: 2/5; subset 2: 2/5; subset 6: 1/5; subset 7:1/5). The NMF of IGHV ongoing mutations significantly decreased from CLL (mean: 0.95×10^{-3}) to RS (mean: 0.17×10^{-3}) (P=0.002). All CLL phases displayed ID (CID: 6/10; UID: 4/10), that, at RS transformation, was switched off in 5/10 cases. ID-positive RS carried a significantly higher number of genetic lesions (mean: 3) than ID-negative RS (mean: 1) (P=0.045). Genealogic trees showed that RS and CLL clones originated from a common progenitor cell in 9/10 cases, whereas in 1 single case RS evolved from a later CLL subclone (Figure 1). Clonally unrelated RS were classified as unmutated (2/3) or minimally mutated (1/3), and carried stereotyped HCDR3 in 1/3 cases (subset 1). All 3 clonally unrelated RS carried CID. Accordingly, the NMF was significantly higher in clonally unrelated RS (mean: 1.0×10^{-3}) compared to clonally related RS (P=0.001). Finally, ID in RS was similar to ID of *de novo* DLBCL sharing

the same non-GCB profile (P>0.050). *Conclusions.* The conclusions of our study are multifold. First, differences in ID reinforce the notion that clonally related and clonally unrelated RS are distinct biological entities. Second, clonally related RS originate from the same common progenitor of the CLL clone, rather than evolving from a later CLL subclone. Third, among clonally related RS, the association between ID and high load of oncogenic molecular lesions might reflect the predisposition to genetic instability of RS cases targeted by somatic hypermutation. In this setting, accumulation of genetic lesions might favor transformation. Fourth, in clonally related ID-negative RS, the tumor clone carries few genetic lesions and is selected to maintain the BCR, suggesting a role of high affinity antigen stimulation in disease development.

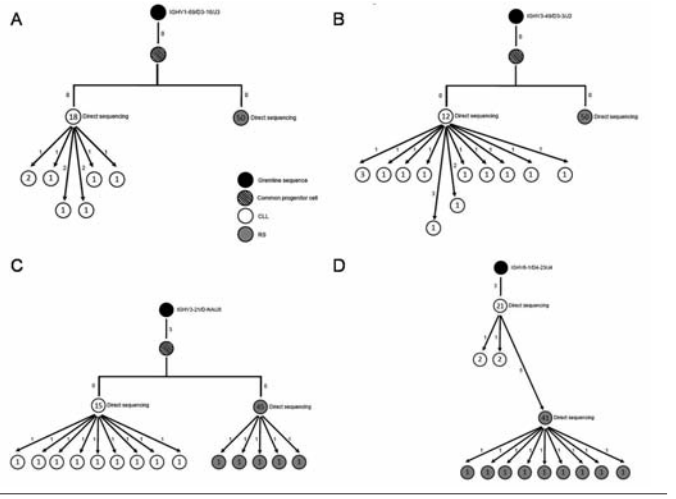

Figure 1.

0107

IGHV1-69/D3-16/J3 SUBSET 6 IS ASSOCIATED WITH INDOLENT DISEASE COURSE OF EARLY STAGE CLL (RAI 0) INDEPENDENT OF UNMUTATED STATUS

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Background. IGHV1-69 gene identifies the most common IGHV gene in chronic lymphocytic leukemia (CLL) and is dominant in unmutated CLL (U-CLL). It is often rearranged to form stereotyped HCDR3 patterns that may suggest antigen selection of the leukemic clones. One of stereotypes is the IGHV1-69/D3-16/J3 subset 6 that may produce antibodies binding non-muscle myosin on apoptotic cells, with potential consequences on clinical behavior (Chu CC, Blood 2010, prepublished). *Aims.* to investigate the prognostic significance of mutational status and of stereotypic B-cell receptors in IGHV1-69$^+$ CLL. *Methods.* Nucleotide sequences of the tumor IGHV1-69/D/J rearrangement, clinical and molecular prognostic parameters at diagnosis and clinical status at follow-up of 271 IGHV1-69$^+$ CLL patients were obtained from 14 different hematological institutes in Italy. CLL B-cell derived IGHV1-69 rearrangements were scanned for HCDR3 stereotypic patterns and assigned to subsets according to the criteria by Murray *et al.* (Blood, 2008;111:1524533). Time to progression requiring first treatment according to NCI criteria (TTFT) was used as the primary endpoint to measure behavior of all or of Rai stage 0 CLL, with mutated (M) or unmutated (U), stereotyped or not stereotyped IGHV1-69 rearrangements. *Results.* Of 271 IGHV1-69$^+$ CLL, 245 (90,4%) were unmutated, 154/257 (59,9%) revealed stereotypic patterns and 15/257 (5.8%) belonged to subset 6. Subset 6 IGHV1-69 rearrangements were unmutated in 14/15 (93.3%) CLL. TTFT was significantly shorter in IGHV1-69+ U-CLL than in IGHV1-69+ M-CLL (29 vs 101 months, P=.001 in all IGHV1-69+ CLL; 52 vs 142 months in Rai 0 IGHV1-69+ CLL, P=.001), while was no different between the CLL assigned or not assigned to subsets (31 vs 37 months in all IGHV1-69+ CLL, 52 vs 78 months in IGHV1-69+ Rai 0 CLL, P=NS). However, specific analysis of the CLL-specific subset 6 revealed TTFT longer than the remaining IGHV1-69$^+$ U-CLL either in all stages (52 months vs 27 months, P=.05) or in stage 0 (no events vs 49 months, P=.016). We expanded the analysis to all IGHV1-69+ and non IGHV1-69+ subsets 1, 3, 8, 9 and 28 (n=50) that also react with autologous non-muscle myosin (Chu CC, Blood, 2010, prepublished). Most remarkably, we found that TTFT of subset 6 was longer than the other anti-myosin subsets (26 months in all stages, P=.021; 44 months in Rai stage 0, P=.02). *Conclusions.* our analysis documents and confirms that unmutated status of IGHV, and not stereotypy, is a relevant prognosticator in CLL. However, the good prognosis of Rai 0 U-CLL assigned to subset 6 suggests a differential clinical behavior irrelevant of unmutated status of this specific subset, particularly if identified in early stage (Rai 0) CLL.

0108

PROGNOSTIC RELEVANCE OF IN VITRO RESPONSE TO CELL STIMULATION VIA SURFACE IGD (SIGD) AND IGM (SIGM) IN BINET STAGE A CRONIC LYMPHOCYTIC LEUKEMIA (CLL)

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Background. Most CLL clones express both sIgM and IgD and the two isotypes share the same antigen-specific combining site. Evidence collected so far supports the notion that both sIgM and sIgD actively participates in the process of clonal expansion since the neoplastic cells could be continuously stimulated *in vivo* possibly by self antigens. *Aims.* Since cell stimulation implies the existence of a viable BcR, we have investigated the effect of stimulating CLL cells *in vitro* by cross-linking their sIgM. The data were correlated with clinical features to investigate a potential involvement of cell stimulation in clonal expansion and disease progression. *Methods.* CLL B-cells, purified from 106 patients, were exposed to aμAb *in vitro* and then apoptosis was measured after 48hrs by tested by annexin V apoptosis assay. Patients were subdivided into three groups, depending upon the type of response observed (inhibition, I; death, D; and null response, N). *Results.* The risk of treatment start was significantly higher ingroup D cases (68 cases, H.R. 2.3, 95% C.I. 1.1-4.9, P=0.038) than in the remaining cases (N+I) (H.R.=1). We previously reported the predictive role of the *in vitro* response to aδ-Ab in the same cohort of patients (Morabito F, Br J Haematol 2009). Out of the 68 cases belonging to group D by aμ-Ab cross-link, 44 showed a null response to aμ-Ab cross-link (N cases), 16 were rescued from apoptosis (I cases), while only 6 cases were also induced to die by aδ-Ab cross-link. Response to aμAb (H.R. 2.8, 95% C.I. 1.2-6.3, P=0.012) remained an independent prognostic indicator also when aδAb was considered (H.R. 2.7, 95% C.I. 1.4-5.5, P=0.012). When patients were stratified according to the response to the two stimuli, 24 cases responding to both stimuli (D and I cases for IgD cross-linking and D cases for IgM cross-linking)) experienced a significantly shorter TFS (HR 6.4, 95% C.I. 1.8-22.6), than cases responding only to aμAb (44 cases) or to aδAb (17 cases). The latter tow patient groups behaved like cases patients with an N response to aδAb or an N or I response to aμ-Ab. After adjustment for CD38 and ZAP-70 expression, and IgVH mutational status, only the response to BCR receptor engagement retained a statistically significant association with the risk of starting treatment. Finally, to investigate whether the groups outlined above showed differences in their gene expression pattern, 70 samples were profiled on Affymetrix HG-

U133A arrays and a multi-class supervised analysis using SAM was carried out on the four groups. We identified 143 differentially expressed genes (150 probes) which specifically distinguished the group responding to IgD from the remaining groups. The 113 genes resulting as preferentially upregulated in the IgD responders with respect to the other 3 classes were mainly involved in intracellular signaling cascade functions (21/113), whereas protein transport (7/30) and transcription (4/30) were found among the negatively modulated functions. *Summary/Conclusions.* Response to sIgD and sIgM cross-linking may represent an additional predictor of disease progression and mayprovide useful information on the mechanisms potentially leading to disease progression.

0109

BRUTON'S TYROSINE KINASE INHIBITORS TARGET B CELL RECEPTOR- AND NURSELIKE CELL-DEPENDENT ACTIVATION OF CHRONIC LYMPHOCYTIC LEUKEMIA B CELL

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Background. Bruton's tyrosine kinase (Btk) is emerging as a therapeutic target in chronic lymphocytic leukemia (CLL). Btk plays an important role in the B cell receptor (BCR) signaling pathway and the development and homeostasis of normal B-lymphocytes. *Aims.* We investigated the effect of three different Btk inhibitors on CLL cell activation after BCR triggering with anti-IgM. Also, these compounds were assessed in CLL co-cultures with nurselike cells (NLC). The compounds disrupt the BCR signaling pathway by selectively targeting Btk (AVL-292 and Compound A) or by targeting Btk and Lyn (Compound B). *Methods.* Viability of CLL cells was determined after 24, 48 and 72 hours of anti-IgM stimulation or NLC co-culture in the presence or absence of the compounds by staining with DiOC6 and propidium iodide (PI). Concentrations of the chemokines CCL3 and CCL4 in CLL cell supernatants after BCR triggering or after co-culture with NLC was analyzed by ELISA. CCL3 and CCL4 are secreted by CLL cells in response to BCR triggering and in NLC-cocultures and function as surrogate markers for BCR responsiveness (Blood 114:1029-37, 2009). The inhibition of Btk phosphorylation was demonstrated by immunoblotting for P-Y223 Btk. To analyze the specific interaction of Btk with the different compounds, a Btk occupancy assay was performed.

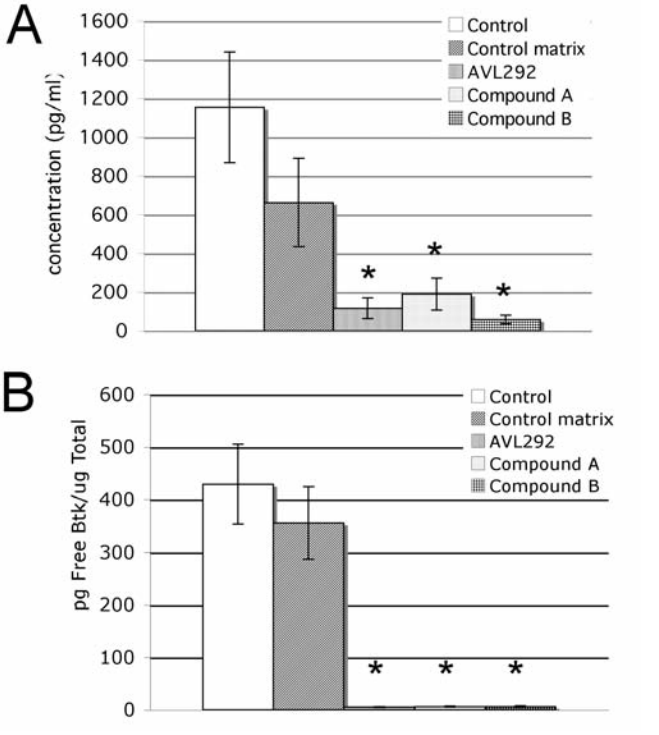

Figure 1. (A) CCL4 expression (B) compound-free Btk protein.

Results. 24 hours after BCR stimulation with anti-IgM, CLL cell viability was significantly reduced by the Btk/Lyn inhibitor Compound B (53.6±6.0% compared to 81.1±3.5% in controls, n=8). Compound B also reduced CLL cell viability in NLC co-cultures (41.6±8.3% compared to 69.5±7.7% in control at 24h, n=8). Both Btk-specific inhibitors were less effective in causing CLL cell death under these conditions, and decreases in viability with these agents were not significant. As expected, high levels of CCL3 and CCL4 were detected in CLL supernatants after 24h of incubation with anti-IgM. Induction of these chemokines was significantly inhibited by Compound B (CCL3: 8.5±0.7 pg/mL versus 1913±76 pg/mL in the control; CCL4:45.1±3.5 pg/mL compared to 4122±159 pg/mL in the control). The other inhibitors had no significant effects on the secretion of CCL3/CCL4 after anti-IgM stimulation. In co-culture with NLCs, the secretion of CCL3 and CCL4 was significantly inhibited by all compounds as shown in Figure 1 A (CCL3: 42.8±27.9 pg/mL with AVL-292, 65.2±19.5 pg/mL with Compound A, 42.8± 27.9 pg/mL with Compound B, compared to 471.5± 160.6 pg/mL in the controls; CCL4: 116.1±52.7 pg/mL with AVL-292, 187.9±82.7 pg/mL with Compound A, 58.4±22.3 pg/mL with Compound B, compared to 1154.2± 286.8 pg/mL in the control, n=6). Phosphorylation of Btk on Y223 was inhibited by both Btk-targeting compounds (AVL-292, Compound B). The occupancy assay revealed complete occupation of Btk by all compounds compared to the control (Figure 1 B). *Conclusions*. Our data demonstrate that the tested compounds are effective in inhibiting BCR- and NLC-derived activation signals in CLL cells. Differences in efficacy of the specific Btk inhibitors versus the Btk/Lyn inhibitor indicate that there is a differential responsiveness to Btk inhibition, depending on the mode of BCR activation. Supra-physiologic BCR triggering by anti-IgM is less responsive to selective Btk inhibitors. In contrast, these Btk-selective agents were extremely effective in blocking NLC-induced CCL3/4 secretion. Because NLC may represent a more relevant mode of CLL cell stimulation than anti-IgM, and because of the exciting therapeutic activity of another Btk inhibitor (PCI-32765) in CLL, these findings indicate that the tested inhibitors represent promising therapeutic agents for CLL patients, alone or in combination with other active agents.

0110

BCR-SIGNALING PROFILES ASSOCIATED WITH PROGNOSIS AND PROGRESSION IN B-CLL

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Background. B-cell chronic lymphocytic leukemia (B-CLL) patients exhibit a variable clinical course. Several biological parameters have been shown to be associated with clinical outcome in CLL. Among them, the most reliable markers are represented by the absence of somatic mutations within the immunoglobulin variable heavy chain genes (IGHV), the expression of CD38 antigen, or the presence of the ZAP-70 tyrosine kinase. These parameters of poor clinical outcome are structurally and/or functionally linked to B-cell Receptor (BCR) expressed by CLL cells, thereby strengthening the hypothesis that antigenic stimulation mediated by the BCR represents a driving event in the onset and progression of the malignant B cells. *Aims.* We investigated whether different BCR signaling networks may distinguish clinical-biological groups of CLL patients. *Methods.* We applied a "network level"analysis of BCR signaling by measuring single-cell profiles of phosphoprotein networks by flow cytometry. We evaluated the response to BCR engagement in primary cells isolated from 27 CLL patients by analyzing the phosphorylation states of 5 phosphoproteins on the route of BCR signaling, including p-Syk, p-NF-kappaB, p-Erk1/2, p-p38 and p-JNK. BCR was cross-linked by incubating cells with anti-IgM antibodies. *Results.* Unsupervised clustering analysis distinguished BCR response profiles of phosphoproteins that differentiated cases of CLL with mutated IGHV from those with unmutated IGHV (P=0.0003), cases with low levels of CD38 expression from those with high levels (P=0.0004) and cases with ZAP-70-negative leukemic cells from cases that were ZAP-70-positive (P=0.001). Furthermore, the same BCR response profiles were also associated with time to progression (P=0.0014) and with overall survival (P=0.049), as assessed by Kaplan-Meier curves and the log-rank test. Independent survival analysis of time to progression via fitting Cox proportional hazards models comprising clinical covariates and/or BCR network response to modulation demonstrated that measuring modulated BCR network signaling can

yield improved prognostic information compared to CD38 status alone (likelihood ratio test 5.8 for CD38 versus 10.6 for signaling) and enhance prognostic assessment using IGHV status (likelihood ratio test for IGHV = 14.8 versus for IGHV + signaling = 17.9). *Conclusions.* This study shows that single-cell profiles of BCR phosphoprotein networks are associated with prognostic parameters, disease progression and overall survival in CLL.

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0111

THE INFLUENCES OF B-CELL RECEPTOR STIMULATION AND ZAP70 EXPRESSION ON MRNA AND MIRNA EXPRESSION PROFILES IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Prognosis of B-Chronic Lymphocytic Leukemia (CLL) is variable, but can be predicted by prognostic markers. The most commonly used prognostic markers include the presence or absence of certain genetic aberration, the mutation status of the immunoglobulin variable heavy chain genes and the expression level of ZAP70. MicroRNA's (miRNAs) are small non-coding RNA molecules that regulate protein expression by targeting the mRNA of protein-coding genes resulting in repression of translation. miRNAs have been shown to be involved in cancer, apoptosis and cell metabolism. In CLL, miRNA expression profiling studies revealed different miRNA profiles associated with known prognostic factors. Since this association is not mechanistically explored and little is known about the regulation of miRNA expression, we investigated the influence of B-cell receptor (BCR) stimulation and ZAP70 protein expression on the miRNA expression profile and interrelate this with a mRNA expression profile. Recently, we demonstrated that clinically significant genes such as ZAP70 could be efficiently overexpressed in CLL cells by electroporation of mRNA (Van Bockstaele F. *et al.* Leukemia 2007). After electroporation we stimulated the cells with anti-IgM-polyacrylamid beads to activate the BCR-signaling pathway and to activate the introduced ZAP70 protein. As a negative transfection control we electroporated uncapped mRNA that remains untranslated in the cell, anti-IgA-polyacrylamid beads (anti-IgA) were used as negative control for stimulation. We determined the miRNA expression profile using a megaplex TaqMan miRNA assay (Mestdagh P. et al. Nucleic Acids Res 2008) together with a Human Illumina Gene Expression beadChip to investigate the differences in mRNA profile. After stimulation, expression of many miRNAs is affected. The most significant differences are the upregulations in hsa-mir-132, hsa-mir-146a and hsa-miR-222. A known target of hsa-miR-146a is IRAK-1 (IL-1 receptor-associated kinase 1). In our micro-array we do see a downregulation after 24 hours of this mRNA and with the use of a real-time qPCR we could confirme these *Results.* Besides IRAK-1, the influenced genes are involved in chemotaxis, cell signalling and cell survival. ZAP70 has no influence on miRNA expression, except for those expressed at very low level. The effect of ZAP70 expression on mRNA profiles will be determined. After stimulation we see that a lot of miRNAs and mRNA genes are regulated. Stimulation of the B-cell receptor activate the NF-kB pathway and this pathway is responsible for at least two different expressed miRNAs. In the future we will further explore the role of these miRNAs and we will try to correlate some differences in miRNA expression with the mRNA expression profile. Further, our data suggest that aberrant ZAP70 expression have no influences on miRNA expression profiles in CLL.

0112

CONSTITUTIVE LEVELS OF PHOSPHORYLATED ERK 1&2 PREDICT OVERALL SURVIVAL AND TIME TO FIRST TREATMENT IN CHRONIC LYMPHOCYTIC LEUKAEMIA

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Background. Chronic lymphocytic leukaemia (CLL) is a clinically heterogeneous disease characterised by an accumulation of malignant B lymphocytes. Central to the survival of CLL-cells is the B-cell Receptor (BCR), a structurally complex molecule which contains an immunoglobulin molecule with antigenic specificity. The Extracellular Regulated Kinase isoforms are active when phosphorylated forming an important part of the intracellular signal transduction mechanism linking the BCR to effector mechanisms such as nuclear transcription factors. Increased levels of phosphorylated Extracellular Regulated Kinase (pERK) are found in CLL cells induced to proliferate and inhibition of the ERK pathway promotes chemotherapy induced apoptosis in B-cell lines. *Aims.* We investigated the relationships between pERK isoforms 1&2 (pERK1&2) concentrations and overall survival (OS) as well as time to first treatment (TTFT) in a cohort of patients with CLL. *Methods.* Lymphocytes were isolated from patients with CLL attending Haematology Clinic at the Hull and East Yorkshire NHS Trust. Collections were made after taking informed consent according to the Declaration of Helsinki with Local Ethics Committee approval (05/Q1104/33). pERK1&2 concentrations were measured quantitatively using ELISA according to the manufacturers protocol (#SUV1018, RnD Systems) in 99 such cases. The mutational status of the variable region of the immunoglobulin gene (IgVH status) was determined in all cases. The pERK1&2 concentration measured was correlated with clinical stage, IgVH, ZAP70 and CD38 status. The pERK1&2 concentration measurements were then ranked and those in the upper quartile were regarded as being elevated. Statistical analysis was performed to assess the relationships between an elevated pERK1&2 concentration and OS / TTFT. *Results.* pERK1&2 concentrations showed a statistically significant relationship with Binet and modified Rai stage (P=0.014 and P=0.049 respectively). There were no correlations between pERK1&2 concentration and CD38 or ZAP70 status (P=0.582 and P=0.609 respectively). A correlation with IgVH status (P=0.038) was detected. An elevated pERK1&2 concentration in the upper quartile of measured results was found to predict a reduced OS (P=0.043). When sub-analysis was performed with stratification by IgVH status the predictive power of an elevated pERK1&2 concentration for OS remained in the IgVH mutated cohort (P=0.011) but not in the IgVH unmutated cohort (P=0.887). An elevated pERK1&2 concentration was also found to predict TTFT (P=0.050). Cases with an elevated pERK1&2 concentration had a median TTFT of 8 months compared to 31 months for those with a low concentration. Sub-analysis by CD38, ZAP70 and IgVH status showed no confounding effects (P=0.566, P=0.898 and P=0.346 respectively). *Conclusions.* Our results demonstrate that increased intracellular pERK1&2 concentration can be used to predict reduced OS and TTFT. An unmutated IgVH status was associated with higher absolute pERK1&2 concentrations suggesting that increased intracellular signalling activity may be one mechanism which links IgVH status and prognosis. The findings suggest that inhibition of the pERK pathway may offer a novel therapeutic approach to the management of bad risk CLL.

0113

DEMONSTRATION OF PHARMACODYNAMIC TARGET INHIBITION AND CHEMOKINE MODULATION IN PATIENTS WITH CLL FOLLOWING TREATMENT WITH CAL-101, A SELECTIVE INHIBITOR OF THE P110 DELTA ISOFORM OF PI3K

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Background. It is well established that deregulation of the PI3K signaling pathway plays an important role in the etiology of human hematologic malignancies. PI3K pathway signaling is mediated by the Class I isoforms (α, β, δ, and γ). We have previously demonstrated that in primary samples from patients with chronic lymphocytic leukemia (CLL), that both constitutive PI3K pathway activation as well as induced activation in response to microenvironmental factors are PI3Kδ-dependent and promote tumor cell survival. CAL-101 is a highly selective and potent p110δ inhibitor (EC50 of 65 nM in a whole blood assay) with >200-fold selectivity over the other class I PI3K isoforms and no activity against Class II and III PI3K family members or other PI3K-related proteins including mTOR and DNA-PK. *Aim.* In an ongoing Phase 1 study enrolling patients with CLL, we sought to determine the effects of CAL-101 treatment on PI3K signaling and to identify evidence of both pathway activation and effects on the tumor microenvironment. *Methods.* Phospho-AKT expression in CLL cells was measured by flow cytometry using antibodies specific for pAKTS473 and pAKTT308. Plasma concentrations of CCL3, CCL4 and CXCL13 were measured by ELISA. *Results.* Serial blood samples from CLL patients with circulating leukemic cells were collected during the clinical trial. Prior to CAL-101 dosing, a high level of constitutive pAKTT308 was observed in all

patients CLL cells (n=11) whereas pAKTS473 was only seen infrequently. At the dose levels evaluated (100 and 150 mg BID) inhibition of constitutive pAKTT308 was nearly complete at 4 hours post dosing on Day 1 and prior to dosing on Day 8. Activation of CLL cells *in vitro* by BCR crosslinking results in the production of CCL3 and CCL4 that was inhibited by CAL-101 treatment. Average plasma concentrations of CCL3 (160 pg/mL) and CCL4 (274 pg/mL) are increased approximately 5-fold in patients with CLL (n=17) compared to normal subjects. In CLL patients treated (n=17) with CAL-101, the concentrations of CCL3 and CCL4 were decreased by 2 to 5-fold in the first week of dosing across all dose levels evaluated (range 50 to 350 mg BID). The B-cell chemokine CXCL13 has been implicated as a pro-survival factor in the interaction between CLL cells and the tumor microenvironment. The average plasma concentration of CXCL13 (240 pg/mL) was 10-fold higher in CLL patients compared to normal subjects. CAL-101 treatment decreased CXCL13 plasma concentration 2 to 5-fold in the first week of dosing across all dose levels evaluated. *Conclusions.* We demonstrate that treatment of CLL patients with CAL-101 results in PI3Kδ target inhibition and reduction of disease-related chemokines produced by both the CLL cells and the tumor microenvironment. These effects are consistent with evidence of clinical activity as measured by a decrease in lymphadenopathy and provide potential biomarkers for drug effect and anti-tumor activity.

0114

MECHANISMS OF ACTION OF TYPE-I AND TYPE-II CD20 MONOCLONAL ANTIBODIES ARE IMPACTED BY TUMOR CELL LOAD

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Background. CD20 represents a well-established target for immunotherapy of B-cell malignancies and autoimmune diseases, particularly through the chimeric antibody rituximab (RTX) which is most widely used. Recently, we described ofatumumab (OFA), a novel human CD20 monoclonal antibody (mAb), which binds a unique membrane-proximal epitope encompassing both the large and small loop on the CD20 molecule. In October 2009, the US FDA approved OFA, under the trademark Arzerra, for the treatment of fludarabine- and alemtuzumab-refractory chronic lymphocytic leukemia. Development of OFA is continuing in multiple additional indications. RTX and OFA are type I CD20 mAbs for which antibody-dependent cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) have been identified as important *in vivo* mechanisms of action. Type II CD20 mAbs, such as B1 and 11B8, in contrast, have been suggested to primarily induce tumor cell kill via ADCC and induction of apoptosis. Complement-dependent cell-mediated cytotoxicity (CDCC) is an effector mechanism which has not yet received much attention. Tumor cell killing by CDCC is mediated via binding of the complement receptor CR3 to complement deposits on target cells. *Aim*. We now investigated the impact of tumor cell load on the mechanisms of action of type-I and type-II CD20 mAbs. *Methods.* The effector mechanisms of RTX, OFA (type I) and 11B8 (type II) CD20 mAbs were studied in novel well-controlled, intraperitoneal, syngeneic EL4-CD20 tumor models set up in wild type and various knock-out mice. We studied tumor cell killing following a short (24 hours) challenge with relatively low (5×10^5 cells) or high (5×10^6 cells) tumor burden. *Results.* All three mAbs (OFA, RTX and 11B8) were effective against a low burden of EL4-CD20 tumor cells in wild type mice, FcγR knock-out mice, FcγR-signalling deficient mice and CR3 knock-out mice. Depletion of complement by cobra venom factor, however, completely inhibited killing. We did not find any evidence for a role of apoptosis induction. Interestingly, OFA and RTX also effectively killed tumor cells in the high tumor burden models, whereas 11B8 did not. Tumor cell kill by OFA and RTX was abrogated in FcγRIII knock-out (but not FcγRI knock-out) mice, CR3 knock-out mice and after depletion of complement. This indicates that under these conditions complement activation as well as CDCC and ADCC are important for tumor kill. *Summary/Conclusions* CD20 mAbs may employ different mechanisms for tumor killing which vary in importance dependent on tumor load. Complement activation plays a critical role in both low and high tumor burden models. This study supports the hypothesis that cooperation of ADCC and CDCC with CDC may be critical for optimal CD20 mAb efficacy.

0115

OFATUMUMAB TARGETS A CONFORMATIONAL MEMBRANE-PROXIMAL EPITOPE WHICH CONTAINS AMINO ACIDS LOCATED IN THE SMALL AND LARGE LOOPS OF CD20

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Background. CD20 represents a well-established target for immunotherapy of B-cell malignancies. Arzerra® (ofatumumab, OFA) is a novel human CD20 monoclonal antibody currently approved for fludarabine-and alemtuzumab-refractory chronic lymphatic leukemia (CLL). Proposed mechanisms of action for OFA include the induction of cell killing via the Fc-mediated effector functions; antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). *Aim*. Based on previous studies we postulate that OFA's exceptional efficacy in activating these effector functions is related to its recognition of a unique membrane-proximal epitope, comprising amino acids in both the small (7 amino acid) and large (44 amino acid) loop of the CD20 molecule. *Methods*. To map OFA's cognate epitope in detail, we generated mutants via site-directed mutagenesis and performed a structure function analysis. To ensure normal surface expression, we employed a strategy in which we generated hybrid CD20 molecules using small loop sequences derived from the MS4A superfamily of which CD20 is a member, and which includes proteins sharing similar structure and limited sequence homology. *Results.* Thus, a complete substitution of the CD20 small loop almost completely abrogated OFA binding. Binding of rituximab (RTX) was not affected. A similar loss of OFA binding was observed when amino acids were replaced at three positions in the small loop (A74T, I76A, and Y77S), in which Y77, in particular, seemed to be crucial. Binding of OFA was also abrogated by mutations in the large loop (T159K, N163D and N166D). Notably, the cognate CD20 epitope for RTX was completely distinct and RTX binding was not affected by any mutations in the small loop, or other mutations affecting OFA binding for that matter. *Summary/Conclusion*. In conclusion, our data show that binding of OFA, but not RTX, is disrupted by amino acid substitutions in the CD20 small loop. Small loop amino acids A74-I76-Y77 are most important and complement the previously identified large loop amino acids T159-N163-N166 in the OFA epitope. The specific involvement of the CD20 small loop, which comprises only 7 extracellular amino acids, presumably positions OFA very close to the membrane, which allows it to harness effector mechanism-mediated killing most efficiently.

0116

GA101 DISPLAYS HIGHER ANTI-LEUKEMIC ACTIVITY MAINLY TROUGH ENHANCED ADCC IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. The efficacy of anti-CD20 monoclonal antibodies (mAb) is a function of direct cell death (DCD), complement dependent cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC). It is difficult to separate the relative contribution of each mechanism *in vivo*. However, *in vitro* tests may help to delineate the relative contribution of each pathway and would allow researchers to better define the mechanism of each pathway. This would allow clinicians to better define mAb and possibly tailor immunomodulation strategies. *Aims.* To investigate the principle mechanism by which GA101, a new type II glycoengineered CD20 mAb, currently in Phase III clinical trials, exerts its activity against fresh B-cell lymphocytic leukemia (CLL) cells. *Methods.* B cell depletion by either Rituximab (RTX) or GA101 was assessed by a flow cytometric B-cell depletion assay using Ficoll-separated peripheral blood mononuclear cell (PBMC) fraction issued from untreated B-CLL patients (n=5) and healthy donors (n=5) in complement heat-inactivated medium. B-cell depletion from PBMC encompasses DCD/ADCC mechanisms. Same experiments were performed with sorted $CD19^+$ leukemic B-CLL cells (n=3) to assess DCD in the absence of effector cells (no ADCC). In these experiments, 10 million cells/mL were incubated in the presence of RTX (10 µg/mL) or GA101 (10 µg/mL) for 7 days. In addition, conventional Cr51 release assays (to assess pure ADCC) were also performed using B-CLL cell as targets and autologous PBMC as effectors (n = 6, using a low E:T ratio of 0.4/1) from CLL patients who received Fludarabine Cyclophosphamide Rituximab (FCR), to assess autologous ADCC after immunochemothera-

py, an unexplored pre-clinical requisite for any maintenance strategy. *Results.* RTX and GA101 induced low rate of depletion in samples containing purified leukemic B-CLL cells (18% and 15%, respectively), suggesting that these two antibodies displayed low intrinsic DCD against purified B-CLL cells. When used in PBMC fraction from normal donors, RTX and GA101 were more potent, but induced similar depletion of normal B-cells (57% and 56% for RTX and GA101, respectively). However, when used in PBMC fraction from B-CLL patients, GA101 appeared to be much more efficient than RTX, with median leukemic cell clearance as high as 97%, compared to only marginal depletion with RTX (P<0.01). Given CLL cells have significantly lowered CD20 density (mean 19000 Antibodies Bound per Cell vs 81000 ABC in healthy B cells, P<0.001), this data indicates that GA101 efficiency is higher on B-CLL than on normal B cells. Finally, GA101 was also more effective than RTX in autologous ADCC assay (patients' effectors against own CLL cells), with mean CLL lysis of 13.85%, versus 7.34% for RTX and <1% with IgG1 control (P<0.05). *Summary*. This study supports the notion that GA101 harness higher ADCC capacity for CLL patients effector cells (as compared to RTX). In particular, we observed that ADCC is measurable before and even after FCR pre-treatment. Taken together these data argue for the use of this antibody in B-CLL therapy.

0117

LUMILIXIMAB MODULATES PI3-K/AKT PATHWAY AND INDUCES APOPTOSIS IN CLL CELLS

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Background. Chronic lymphocytic leukemia (CLL) is characterized by a defect in apoptosis mechanisms and the high expression of CD23. Lumiliximab is a monoclonal antibody against CD23 which has been shown to exert a promising therapeutic effect in CLL *in vivo* and to induce apoptosis in $CD23^+$ lymphoma cells *in vitro*. *Aims.* The aim of this study was to investigate the molecular mechanism of action of lumiliximab in CLL cells and its role in the regulation of the anti-apoptotic PI3-K/Akt cascade signaling in primary CLL cells. *Methods.* PBMC from CLL patients were exposed to lumiliximab (1-50 µg/mL) for 1-15 days in co-culture with autologous primary human stromal cells which prevent spontaneous apoptosis of CLL cells. Cell viability was assessed by flow cytometric analysis using annexin V/PI staining and by MTT assays. *Results.* The results showed that single exposure to lumiliximab had a moderate effect on cell viability (<1 fold increase in apoptosis rate compared to untreated controls). However, repeated exposure to lumiliximab had a significant pro-apoptotic effect selectively in the $CD19^+/CD5^+/CD23^+$ cells. Western blotting demonstrated that lumiliximab decreased the levels of Bcl-2, Mcl-1 and Hsp70 protein expression. In addition, it resulted in a significant decrease in the phosphorylation of Akt (Ser473) and dephosphorylation of the tumor suppressor PTEN (Ser380) suggesting the involvement of the PI3-K/Akt/PTEN cascade in priming CLL cells to undergo apoptosis by lumiliximab. Pre-incubation of CLL cells with lumiliximab enhanced the pro-apoptotic effect of PI3-K inhibitor LY292004 and the casein kinase-2 (CK2) inhibitor apigenin. Microarray analysis and pathway exploration revealed that lumiliximab regulates several sets of genes which are involved in chemokine signaling, cytokine/cytokine receptor interaction, oxidative stress, PI3-K and integrin signaling, complement cascade and Toll-like receptor signaling. *Conclusions.* The data demonstrate that exposure to Lumiliximab is effective in priming CLL cells to undergo apoptosis through inactivation of the PI3-K/Akt pathway. The data also provide further evidence of a promising therapeutic role for lumiliximab in CLL and a rationale for lumiliximab-based drug combinations to improve treatment of this disease and other disorders associated with increased expression of CD23.

0118

ABNORMAL IMMUNOGLOBULIN SERUM FREE LIGHT CHAINS RATIO IS ASSOCIATED WITH UNFAVORABLE PROGNOSTIC MARKERS AND IS A PREDICTOR OF TREATMENT FREE SURVIVAL IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Assessment of sFLC concentrations and ratio is a powerful tool to qualify response stringency and duration in patients with multiple myeloma. The presence of an abnormal sFLC ratio also represents a risk factor for progression in different types of plasma cell dyscrasias. Being a sensitive, simple and highly reproducible indicator of B-cell clonality, the sFLC test is particularly attractive as a putative prognostic marker in CLL as compared with current assays including CD38 or ZAP-70, FISH analysis, or evaluation of IgVH gene mutational status. *Patients and Methods*. To assess the prognostic value of sFLC testing in CLL, 248 untreated CLL (Binet stage A=162, B=26, C=44) patients (138 males; median age 64 yrs) were enrolled in a collaborative retrospective study. TFS was calculated from diagnosis to first treatment. Median follow-up was 2.7 years (range 1-20) and 83 of 232 patients analyzed for clinical outcome received treatment by the end of the study. Cryopreserved (-80°C) serum samples were collected and sFLC levels were determined in the reference study laboratory using a particle-enhanced, high-specificity, homogeneous FLC immunoassay [Freelite; The Binding Site, Birmingham, UK, performed on a Delta Nephelometer (Radim)]. *Results.* ZAP-70, CD38 and IgVH mutational analyses were performed in 217 (80 $ZAP\text{-}70^+$), 226 (47 $CD38^+$) and 178 (62 unmut-IgVH) cases, respectively. FISH in 129 cases: normal (52 cases), 13q14.3 deletion (31), trisomy 12 (15), 11q22.3 (11) and 17p13.1 (17). An abnormal sFLC ratio was found in 85 cases (73κ, 12λ). No significant correlation emerged between sFLC abnormality and Binet stage, while a significant association was demonstrated with CD38 (P<0.0001) and ZAP-70 (P=0.005) expression and IgVH mutation status (P=0.033); FISH results showed no correlation with abnormal sFLC testing. Univariate Cox analysis showed a statistically significant higher risk of treatment initiation in Binet B+C pts (HR=1.6, 95% C.I. 1.1-2.5, P=0.034), $CD38^+$ (HR=2.2, 95% C.I. 1.4-3.5, P=0.002), $ZAP\text{-}70^+$ (HR=5.6, 95% C.I. 3.5-9.3, P<0.0001), unmut-IgVH (HR=3.8, 95% C.I. 2.2-6.4, P <0.0001), and in cases deleted for 17p13.1 and 11q (HR=4.1, 95% C.I. 1.5-5.0, P=0.001). To assess the prognostic relevance of sFLCs, patients were subdivided into normal or abnormal ratio groups. The analysis revealed a significantly higher risk of progressing to treatment for patients with abnormal sFLCs (HR=2.5, 95% C.I. 1.6-4.0, P<0.0001). Notably, sFLCs retained an independent association with TFS even when adjusted for either Binet stage, CD38 or ZAP-70 expression, IgVH mutational status and FISH abnormalities. *Summary/Conclusion.* While the correlation of ZAP-70 and CD38 expression, and IgVH mutational status indicates that an abnormal sFLC ratio could identify patients with a more aggressive subtype of CLL, the lack of association with Binet staging suggest the sFLC ratio is not simply reflecting disease bulk but rather mirrors specific biological features of tumor cells. A prospective confirmation of the predictive prognostic power of the sFLC ratio will be of utmost relevance given that sFLC assay is a simple and highly reproducible test as compared to the more complex determination of IgVH mutation status involving DNA sequencing or ZAP-70 status where significant inter-laboratory variation exists.

Chronic myeloid leukemia - Biology 1

0119

GENOMIC SEGMENTAL DUPLICATIONS ARE INVOLVED IN THE OCCURRENCE OF T(9;22) REARRANGEMENT IN CHRONIC MYELOID LEUKEMIA

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Background. A crucial role of segmental duplications (SDs) of the human genome has been demonstrated in chromosomal rearrangements associated with several genomic disorders. Limited knowledge is yet available on the molecular processes resulting in chromosomal rearrangements in tumors. The t(9;22)(q34;q11) causing the 5'BCR/3'ABL gene formation has been detected in more than 90% of chronic myeloid leukemia (CML) cases. *Aims.* In 10-18% of CML patients genomic deletions were detected on der(9) chromosome next to translocation breakpoints. The molecular mechanism triggering the t(9;22) and deletions on der(9) is still unknown. Our study presents an experimental evidence of the involvement of SDs in the genesis of the t(9;22) translocation in CML and in the occurrence. *Methods.* We studied 71 cases with der(9) deletions by FISH. Fine-mapping of deletions was performed using appropriate bacterial and Phage P1-derived artificial chromosome clones. The mapping of all breakpoints revealed an evident breakpoints clustering, on both chromosomes 9 and 22, in two regions of about 2 Mb in size. Indeed, these regions contained the breakpoints detected in 54 out of 60 (90%) patients bearing chromosome 9 deletions and in all patients with chromosome 22 sequences loss. Bioinformatic analysis of chromosome 9 and chromosome 22 genomic regions involved in the deletions was performed to search for features that could correlate with the breakpoints clustering. To this aim, the breakpoint regions were subdivided into 250 Kb intervals. *Results.* The most striking result was the fact that both clusters contain a previously described 76-kb duplicon, shared by chromosome 9 and 22 (SD_9/22). The SD_9/22 is the only duplication located inside the breakpoints clustering region on chromosome 9, whereas the chromosome 22 clustering region harbors several duplications. A remarkable feature of the chromosome 9 clustering region was the high frequency of Alu repeats. The mean Alu frequency overall on chromosome 9 is 10.8%, whereas the average Alu content on this cluster is 31.3%. Accordingly, as expected, the content in LINE sequences of the region was relatively low. Gene distribution analysis of chromosome 9 and 22 showed that both SD_9/22 map inside gene-poor regions. A statistically significant negative association was observed between the number of breaks and the distance from SD_9/22, on both chromosomes 9 (P=0.01) and 22 (P=0.006), respectively. The relationship between the breaks and the interspersed repeats revealed, on chromosome 9, a positive linear regression with Alu repeats (P=0.04), and a negative one with LINEs (P=0.04). Very similar conclusions were obtained by comparing the distance from the SD_9 and the Alu (P= 0.03, positive) and LINE distribution (P=0.02, negative). No statistically significant relationship was observed on chromosome 22. *Conclusions.* In our study the involvement of SDs was proposed to explain the recurrent t(9;22) translocation in CML and the genomic deletions that could accompany the rearrangement. At the light of these findings, the analysis of secondary non-recurrent events could represent a new methodological approach able to identify architectural elements involved in the occurrence of recurrent primary rearrangements in human neoplasia.

0120

FOXO TRANSCRIPTION FACTOR ACTIVITY IS PARTIALLY RETAINED IN QUIESCENT CML STEM CELLS AND INDUCED BY TYROSINE KINASE INHIBITORS IN CML PROGENITOR CELLS

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Background. Chronic Myeloid Leukaemia (CML) is a clonal disease initiated and maintained by the tyrosine kinase BCR-ABL. BCR-ABL signaling activates the PI3K/AKT pathway (amongst others), leading to inhibition of the transcriptional activity of the FOXO family proteins. ABL-specific tyrosine kinase inhibitors (TKIs), whilst effective against mature CML cells, induce little apoptosis in stem/progenitor cells. In addition, TKIs exert potent anti-proliferative effects in stem/progenitor cells through a poorly understood mechanism. FOXO transcription factors are known to transcriptionally modulate proteins that induce cell cycle arrest. Therefore, BCR-ABL-mediated inhibition of FOXO may contribute to CML cell proliferation and malignant transformation. *Aims.* To investigate the mechanism of TKI-induced G1 arrest in primary CML stem/progenitor cells in relation to FOXO proteins. *Methods.* We performed *in vitro* experiments by flow cytometry analysing phosphorylation of FOXO1, 3a and 4 in CML $CD34^+$ and $CD34^+38^-90^-$CML cells treated with 150 nM dasatinib for 24 h. Western blotting and immuno-fluorescence were used to show relocation of total FOXO1, 3a and 4 from the cytoplasm (inactive) to the nucleus (active) after dasatinib treatment. *Results.* We showed that in $CD34^+$ CML cells FOXO1, 3a and 4 (FOXOs) were phosphorylated, predominantly cytoplasmic and inactive, consequent to BCR-ABL expression. TKIs, such as dasatinib, decreased phosphorylation of FOXOs, leading to their re-localisation from cytoplasm to nucleus, thus inducing cell cycle arrest. Interestingly, despite BCR-ABL activity, primitive quiescent CML stem cells ($CD34^+38^-90^-$) showed low levels of FOXO phosphorylation, resembling the pattern in normal stem cells. *Conclusions.* These results demonstrate that TKI-induced G1 arrest in CML progenitor cells is mediated by re-activation of FOXOs, whilst quiescence of CML stem cells is, at least in part, regulated by sustained FOXO activity. These data contribute to our understanding of CML stem cell quiescence and TKI activity, suggesting new strategies to target CML stem/progenitor cells by preventing or reversing this effect.

0121

ABT-737 COOPERATES WITH TYROSINE KINASE INHIBITORS TO INDUCE APOPTOSIS OF CHRONIC MYELOID LEUKEMIA CELLS THROUGH BCL-XL INHIBITION AND IAP PROTEINS DOWN-REGULATION

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BCR-ABL confers an apoptosis-resistant phenotype due to its kinase activity. Several pro- and anti-apoptotic proteins of the Bcl-2 family modulate the apoptotic signal. Amongst them, anti-apoptotic Bcl-xL and Mcl-1 are induced by BCR-ABL. Conversely, Bim expression strongly affects tyrosine kinase inhibitor-induced apoptosis and Bim-depleted cells were unable to undergo apoptosis when treated by either imatinib (IMA) or nilotinib (NIL). It has been shown that Bim actually is an indirect activator of apoptosis through its anti-Bcl-xL, -Mcl-1 and -Bcl-2 effects. Recently, ABT-737, a small molecule which binds Bcl-2 and Bcl-xL but not Mcl-1, was shown to induce apoptosis in several tumour cell types. In this study, we verify if ABT-737 could cooperate efficiently with the Bim stabilizing TKI. The ABT-737 ability to displace the Bcl-2 family proteins interaction was investigated by co-immunoprecipitation on K562 cells. ABT-737 decreased the BIM/Bcl-XL interaction in cell free system as in intact cells and increased the BIM/MCL-1 interaction supporting the complementary effects of TKI (stabilizing Bim) and ABT-737. K562 cells were incubated with increasing concentrations of either imatinib or nilotinib, alone or in combination with ABT-737, in a constant ratio. Both TKI and ABT-737 induced a dose dependant apoptosis and the combination of both TKI with ABT-737 resulted in a synergistic cooperation to induce apoptosis as assessed using the Calcusyn software. Similar experiments were performed on CD34 expressing cells from 12 CML patients, and the strong synergism of ABT-737 with both TKI was confirmed. To elucidate the mechanisms underlying this cooperation, we analyzed the Bcl-2 family proteins expression and confirmed the increase of Bim and the decrease of Bcl-xL due to TKI treatment while ABT-737 remained without effect. The expression of several anti-apoptotic proteins was also analyzed. Surprisingly, the TKI/ABT-737 association induced a strong decrease in two IAP family members: XIAP and cIAP1. This decrease was accompanied by an increase in caspase 3 activation. Cell sorting experiments showed that the IAP decrease preceded the drop in mitochondrial membrane potential and caspase 3 activation. This led us to investigate the role of the IAP family proteins in the TKI treatment response by using the LBW-242, a Smac mimetic inhibitor of IAPs. K562 cells were incubated with imatinib alone or in combination with LBW-242, in a constant ratio. Both IMA and LBW-242 induced a dose dependant apoptosis and the combination of IMA with LBW-242 resulted in a synergistic cooperation to induce apoptosis. Similar experiments with similar results were performed on CD34 expressing cells from 10 CML patients, confirming the adverse effect of IAPs on TKI-induced apoptosis. The ABT-737/TKI cooperation to induce apoptosis of CML cells can be explained by the increase in Bim content induced by TKI associated to the inactivation of Bcl-2, -xL

by ABT-737. However, a side effect at the level of the caspase inhibitors XIAP and cIAP1 also seems to participate, resulting in a strong synergism. Thus, the association of BH3- and/or Smac-mimetics with TKI treatment could be a facilitating strategy to induce apoptosis in some BCR-ABL expressing cells resistant to TKI alone.

0122

HIGH-RESOLUTION MOLECULAR KARYOTYPING OF CHRONIC MYELOID LEUKEMIA PATIENTS IN BLAST CRISIS BY 6.0 SNP-ARRAYS IDENTIFIES FOCAL COPY NUMBER ALTERATIONS AFFECTING ONCOGENES AND TUMOR SUPPRESSOR GENES

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Background. Despite the striking efficacy of targeted therapy for chronic myeloid leukemia (CML), a proportion of patients (pts) still experience progression from the initial chronic phase to an acute phase (blast crisis; BC) characterized by high disease aggressiveness and poor prognosis. BC is known to be associated with accumulation of additional genetic alterations, but these alterations have so far been only partially characterized. *Aims.* We have used Human 6.0 SNP Arrays (Affymetrix) to perform high-resolution molecular karyotyping of 30 DNA samples from BC (myeloid, n=23; lymphoid, n=7) CML pts. *Methods.* The 6.0 SNP Array technology relies on 1.8 million markers evenly spaced across the genome, with a median inter-marker distance <700 bp. So, we decided to exploit the unprecedented resolution of Human 6.0 SNP Arrays and the ability of Genotyping Console 3.0.2 (Affymetrix) software to pinpoint the borders of these CNAs. We thus aimed our analysis to the identification of very small CNAs that may have been missed by previous studies - all using less sensitive assays. Gains/losses mapping to known regions of copy number variation (CNV) were excluded. *Results.* Our approach revealed a number of focal gains or losses ranging from 4 to 47Kb, affecting a single gene or, more frequently, only part of a gene. Amplifications were as frequent as monoallelic deletions and involved the promoter region and or one or more exons. In some cases, a complex pattern of amplification of some exons and monoallelic deletion of others was recognized within the same gene. These alterations were found in 2 to 12 pts each, and involved the following genes: AKT3; CDC73; RB1; JAK2; JAK1; ERG; ETS1; SMAD; PIK3CA; EPHA3; RUNX1T1; ETV1; AKT2; MDM4; KALRN; FHIT; K-RAS; PTEN; FAF1; SKAP2; PTCH1; GAS2; FGFR2; SOS1; NRG1; MET; PBX4; ETV5; N-RAS, HGF, TEC; PAK2; H-RAS. The precise anatomy of alterations will be presented. Deeper characterization at the DNA and RNA level by polymerase chain reaction and sequencing is ongoing. All the genes identified in our screening were transcription factors, adaptor proteins, receptor and non-receptor kinases involved in cell proliferation and apoptosis - with a known role as oncogenes or tumor suppressors or oncogene/tumor suppressor interactors. *Conclusions.* Our results confirm a high degree of heterogeneity in the alterations detectable in BC CML pts. In conclusion, the power of 6.0 SNP Array technology allowed us to detect previously unidentified alterations targeting whole or part of key oncogenes or tumor suppressors whose deregulation may play a role in determining the aggressive phenotype of BC CML and which may represent potential therapeutic targets. Supported by European LeukemiaNet, AIL, AIRC, PRIN, Fondazione del Monte di Bologna e Ravenna.

0123

NON RANDOM DISTRIBUTION OF GENOMIC FEATURES IN BREAKPOINT REGIONS INVOLVED IN CHRONIC MYELOID LEUKEMIA CASES WITH VARIANT T(9;22) OR ADDITIONAL CHROMOSOMAL REARRANGEMENTS

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Background. The t(9;22)(q34;q11), generating the Philadelphia (Ph) chromosome, is found in more than 90% of patients with chronic myeloid leukemia (CML). At diagnosis, in 5-10% of CML patients the Ph chromosome is derived from variant translocations other than the standard t(9;22). *Aims.* The aim of this study was to perform an accurate breakpoints identification and bioinformatic analysis of other chromosomes involved in variant t(9;22) or in concomitant chromosomal rearrangements apart from the t(9;22). *Methods.* Four hundred and fifty two consecutive CML patients in chronic phase were analyzed by conventional cytogenetic analysis and by FISH experiments with probes specific for ABL and BCR genes. All breakpoints on other chromosomes involved in variant t(9;22) and in additional rearrangements have been characterized by FISH experiments and bioinformatic analyses. Breakpoint regions on other chromosomes involved in variant t(9;22) and additional rearrangements were included in 250 Kb size intervals. Each interval was checked for the presence of interspersed repeats classes (Alu and LINE repeats), segmental duplications (SDs), GC content, gene density, and miRNA. *Results.* The molecular cytogenetic analysis revealed 50 CML cases identifying three main subgroups: i) cases with variant chromosomal rearrangements other than the classic t(9;22)(q34;q11) (9.5%); ii) cases with cryptic insertions of ABL1 into BCR, or vice versa (1.3%); iii) cases bearing additional chromosomal rearrangements concomitant to the t(9;22) (1.1%). Bioinformatic analysis showed that the majority of breakpoints on chromosomes involved in variant or additional chromosomal rearrangements showed a high frequency of Alu repeats. In fact, 41 out of 58 (71%) breakpoints showed an Alu content of more than one whereas the remaining 17 out of 58 (29%) had a content of less than one. Instead, the LINE content was lower than one in 44 out of 58 (76%) breakpoints. Most of the analyzed breakpoints map within gene-rich regions in 45 out of 58 (78%) breakpoints. Moreover, 49 out of 58 (84%) breakpoints revealed a low SDs density. A GC content >1 was detected in 43 out of 58 (74%) breakpoints. The search for miRNAs revealed a different density from the expected value in 33 out of 58 (57%) breakpoint regions. In detail, in 29 (88%) and 4 out of 33 (12%) breakpoints a higher or lower number of miRNA than the expected value was identified, respectively. In the remaining 25 out of 58 (43%) breakpoints no miRNA was revealed in the 4 Mb analyzed intervals. *Conclusions.* This study revealed a high content of Alu repeats, genes density, GC frequency, and miRNAs in the great majority of the analyzed breakpoints, suggesting their potential involvement in the CML pathogenesis. In conclusion, our findings demonstrate that the involvement of chromosomes other than 9 and 22 is not a random event but could depend on specific genomic features.

0124

NAMPT, A MEDIATOR OF NORMAL GRANULOCYTIC DIFFERENTIATION, IS DOWN-REGULATED IN CHRONIC MYELOID LEUKAEMIA VIA CHROMATIN MODIFICATION

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Background. Chronic myeloid leukaemia (CML), a haematological malignancy initiated by the BCR-ABL1 fusion, is characterised by an overproduction of immature granulocytes. The BCR-ABL1 gene fusion is acquired at the stem cell level, but the precise events that govern the proliferation and differentiation of the CML clone are poorly understood. In normal haemopoiesis, the NAMPT gene (also known as PBEF1) was recently shown to mediate G-CSF-induced myeloid differentiation via the NAD pathway, raising the possibility that this gene may be involved in the pathogenesis of CML. *Aims.* To confirm the role of NAMPT in granulocytic differentiation and to investigate the expression of NAMPT in CML. *Methods.* A MIGR1 retroviral construct containing NAMPT was used to transduce murine haemopoietic stem cells (HSCs). Cells were stained with granulocytic marker Gr-1 conjugated with PE and monocytic/macrophage marker Cd11b conjugated with APC and subjected to flow-cytometric analysis. NAMPT expression in granulocytes and CD34 cells from CML patients and normal individuals was assessed by TaqMan-based real-time RT-PCR. Allelic ratio testing was performed by pyrosequencing (for SNPs) or capillary electrophoresis using Genescan fragment analysis software (for the insertion/deletion polymorphism). For assessment of nuclease sensitivity, normal and CML granulocytes were fixed, permeablised and treated with micrococcal nuclease prior to real-time PCR using primers within the NAMPT promoter. The methylation status of the NAMPT promoter was assessed by cloning and bisulphite sequencing. *Results.* In keeping with published data we observed increased granulocytic differentiation in murine haemopoietic stem cells transduced with NAMPT. Interestingly, however, we found that NAMPT levels in granulocytes from chronic phase CML patients at presentation (n=20) were 410-fold low-

er than normal controls (n=30; P<0.0001; CML range 0.7-19.7, normal range 128-4470). NAMPT expression in CD34 cells from CML patients was also low, but not significantly different to expression in normal CD34 cells. We then performed several investigations into the mechanism of NAMPT silencing. Allelic ratio testing using an insertion deletion polymorphism and 3 SNPs within the NAMPT locus revealed no evidence of loss of heterozygosity. Application of a real-time PCR-based quantitative nuclease sensitivity assay revealed decreased nuclease sensitivity in the promoter region of NAMPT in CML granulocytes, but not in normal granulocytes, in keeping with chromatin condensation in this region. There was no evidence of methylation in CpG islands of the promoter by cloning and bisulphite sequencing, suggesting an alternative mechanism of chromatin modification. *Conclusions.* Taken together our findings indicate disease-specific epigenetic silencing of NAMPT in CML. This would be in keeping with the recent suggestion that NAMPT functions as a tumour suppressor gene. Alternatively, downstream BCR-ABL1 signalling pathways may initiate granulocytic differentiation independently of NAMPT, bypassing the stage at which the gene is usually activated.

0125

BCR-ABL BUT NOT JAK2 V617F INHIBITS ERYTHROPOIESIS THROUGH THE RAS SIGNAL BY INDUCING P21CIP1/WAF1

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Background and *Aims.* BCR-ABL is a causative tyrosine kinase (TK) of chronic myelogenous leukemia (CML). In CML patients, anemia is commonly observed in contrast to marked leukocytosis. This phenotype is quite different from that of polycythemia vera caused by JAK2 V617F, although both TKs activate common downstream molecules at the hematopoietic stem cell level. To clarify this mechanism, we investigated the effects of BCR-ABL and JAK2 V617F on erythropoiesis. *Methods.* We introduced BCR-ABL, JAK2 V617F, and active forms of their downstream molecules: N-RasE12 (N-Ras), 1*6 STAT5A (STAT5) and $p110^{CAAX}$ (phosphatidylinositol 3-kinase), into murine bone marrow LSK (Lineage-$Sca\text{-}1^{hi}CD117^{hi}$) cells by retrovirus system. After 5 days of culture with murine stromal MS-5 cells, the fraction of $CD45^{low}TER\text{-}119^{+}$ erythroid cells was evaluated by flow cytometry. *Results.* BCR-ABL reduced the erythroid fraction while JAK2 V617F did not (proportions: Mock, 11.4%; BCR-ABL, 5.4%; JAK2 V617F, 12.0%). Also, BCR-ABL but not JAK2 V617F reduced the number of BFU-E. Among their downstream molecules, 1*6 STAT5A and $p110^{CAAX}$ increased the numbers of both erythroid (2.8- and 1.9-fold, respectively) and myeloid cells (4.9- and 3.0-fold, respectively). On the other hand, N-RasE12 reduced the number of erythroid cells (0.28-fold) in contrast to the significant increase in myeloid cells (3.6-fold). In immunoblotting, ERK was more intensely phosphorylated in BCR-ABL-transfected Ba/F3 cells than in JAK2 V617F-transfected cells, suggesting that different growth status of erythroid cells between these TKs results from the preferential activation of Ras signal by BCR-ABL. As for the mechanism through which Ras promotes myelopoiesis but suppresses erythropoiesis, we examined the effect of GATA-1, a transcription factor expressed in erythroid but not in myeloid lineage, on Ras signaling. In Ba/F3 cells transduced with N-RasE12 and GATA-1/ERT, activation of GATA-1 by 4-Hydroxytamoxifen completely suppressed Ras-dependent growth and survival. Luciferase assays and immunoblotting revealed that GATA-1 blocked the activity of Ras/Raf/MEK/ERK signaling at the level of MEK. In GST pull-down assays, GATA-1 interacted with MEK directly. Together, these data indicate that GATA-1 blocks Ras/Raf/MEK/ERK mitogenic signal through the direct interaction with MEK. Oncogenic Ras, while promoting cell growth, also causes growth arrest to act against malignant transformation. Because this response is mediated by several pathways involving cell cycle regulators such as p53, $p16^{INK4a}$, $p19^{ARF}$ and $p21^{CIP1/WAF1}$, we investigated their roles in Ras-induced suppression of erythropoiesis. Expression of N-RasE12 in LSK cells from p53, p16INK4a/$p19^{ARF}$ or $p21^{CIP1/WAF1}$-wild-type/knock-out mice revealed that the proportion of erythroid cells reduced by N-RasE12 was restored only by $p21^{CIP1/WAF1}$ deficiency (wild-type, 3.0%; knock-out, 5.2%). Also, expression of N-RasE12 in $p21^{CIP1/WAF1}$-knock-out cells increased the erythroid cell number compared with Mock-transduced wild-type and knock-out cells. Although $p21^{CIP1/WAF1}$ is a transcriptional target of p53, our results indicate that Ras suppresses erythropoiesis through $p21^{CIP1/WAF1}$ in a p53-independent manner. *Conclusions.* In Ras-induced suppression of erythropoiesis, GATA-1 blocks growth-promoting signals by direct interaction with MEK, leading to the relative dominance of growth-suppressing signals. We also identified $p21^{CIP1/WAF1}$ as a central mediator in Ras-induced suppression of erythropoiesis. These mechanisms would be helpful for explaining how respective TKs reveal different disease phenotypes.

0126

MIR-451 MAY POSITIVELY REGULATE MDR1 IN CHRONIC MYELOID LEUKEMIA

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MicroRNAs (miRNAs) are small single-stranded RNAs that regulate gene expression by inhibiting translation or by decreasing stability of target mRNAs. Aberrant expression of miRNAs has been associated with some hematological malignancies. Previously, we observed expression changes of miR-451 at different stages of chronic myeloid leukemia (CML) and different responses to imatinib in comparison to healthy control using microRNA arrays. We validated aberrant expression of miR-451 by real-time PCR on the cohort of 116 patient samples of total leukocytes representing: diagnosis (Dg; 100% Ph^{+}) n=15, major molecular response (MMR; <0.1% BCR-ABL) n=26, suboptimal response/therapy failure (SR/TF; 30-100% Ph^{+}) n=29, hematological relapse (Hr; 100% Ph^{+}) n= 24 and accelerated phase together with blast crisis (AP/BC; 10-79% blasts) n=22. Down-regulation of miR-451 was found in 73% (11/15) of Dg, 71% (17/24) of Hr and 55% (12/22) of AP/BC patient samples in comparison to healthy controls (n=11). On the other hand expression of miR-451 was up-regulated in some MMR (12/26) and SR/TF (10/29) patient samples. To explore possible consequences of miR-451 differential expression in response to imatinib, we investigated in this study the expression of multidrug resistant gene (MDR1) that was described to be activating by miR-451 in human cancer cells (Zhu et al., Biochemical Pharmacology 2008,26:582). Using real-time PCR we found down-regulated MDR1 expression on the same cohort of patient samples among healthy control in Dg, Hr and AP/BC, and up-regulated MDR1 levels in MMR and SR/TF, which suggested correlation between miR-451 and MDR1 expressions and supported results of Zhu *et al.* (2008). To confirm our data found *in vivo*, we treated leukocytes of newly diagnosed patients (n=6) with imatinib *in vitro*. Inhibition effect of imatinib was proved by decreased p-Crkl levels (Western blot) and decreased WT1 transcript levels (real-time PCR) (Svensson *et al.,* Leukemia,21:2485). Apoptosis was checked by RNA degradation levels and by annexing V staining to avoid its interference to expression analyses. Also we checked that imatinib free cultivation after 2h, 24h, 48h and 78h did not affect expression of WT1, miR-451, MDR1 and p-CRKL levels. JURL and CML cell lines were used as positive controls and leukocytes of healthy donors (n=3) were used as negative controls. Imatinib naïve patient leukocytes and BCR-ABL positive cell lines expressed down-regulated miR-451 and MDR1 and high BCR-ABL activity. We showed that miR-451 levels and MDR1 mRNA levels increased together with inhibition of BCR-ABL activity in imatinib treated leukocytes. In conclusion, from our expression data *in vivo* and *in vitro* it seems that miR-451 may positively regulate MDR1 expression in CML. Moreover, our data showing increased levels of MDR1 in leukocytes of CML patients in MMR as well as increased levels of MDR1 and inhibited BCR-ABL activity in leukocytes of CML patients treated with imatinib *in vitro* suggested that MDR1 is unlikely to mediate imatinib resistance in CML patients, which is in agreement with Hatziieremia *et al.* (Experimental Hematology 2009;37:692).

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0127

PTCH1: NEW TARGET FOR MONITORING CHRONIC MYELOID LEUKAEMIA AND THERAPY

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Background. Chronic myeloid leukemia (CML) is characterized by the expansion of a leukemic stem cell clone carrying a Philadelphia translocation, which outgrows the non-malignant haematopoietic stem cells. The existence of a leukemia stem cell population may account for drug-resistance and relapse during treatment. The tyrosine kinase inhibitors (TKIs) imatinib, nilotinib and dasatinib, all show impressive rates of complete cytogenetic response in chronic phase (CP) CML.

However, the majority of responding CP CML patients have detectable BCR-ABL transcripts which might arise from a population of quiescent CML stem cells not effectively targeted by TKIs. Recent studies have indicated that the Hedgehog (Hh) pathway, a developmental pathway with roles in primitive and adult hematopoiesis, is activated in a wide variety of human cancers, included CML, via upregulation of Smoothened (SMO), a seven-transmembrane domain receptor protein. Notably several highly conserved members of this pathway are inappropriately activated such as Ptch1 (surface receptor regulator of SMO). *Aims.* Better understanding of the role of Hh signaling in CML, to identify predictive and pharmacodynamic markers of tumour responses. In this study, we identified PTCH1 as a molecular marker in Bcr-Abl-positive leukemic stem cells. *Methods and results.* in order to examine the functional role of Hh signaling in CML, we treated cells line K562 with the naturally occurring inhibitor of SMO, cyclopamine (2-10 µM), alone and with TKIs imatinib (1000-125 nM). After 48h we measure specific cell death staining with propidium iodure and flow-citometry. The inhibition of Hh signaling failed to affect survival of cell lines. Remarkably, imatinib and cyclopamine showed a potent synergistic interaction consistently exhibiting combination indices below 1 upon exposure to a wide range of drugs concentrations (Figure 1). Subsequently, we analyzed the Ptch1 mRNA levels in 20 CP-CML patients (8 high, 4 intermediate and 8 low Sokal risk respectively) on bone marrow samples collected at diagnosis. Using RQ-PCR we founded that PTCH1 is significantly upregulated (3-fold) at the mRNA level in bone marrow $CD34^+$ of CML patients (40%). Surprisingly 6/8 (75%) of high Sokal risk tested showed up-regulation of upstream effector Ptch1 (indicative of active Hh signalling). We also studied normal $CD34^+$ bone marrow cells of healthy donors (n=3) and found that they did not express PTCH1 mRNA level by RQ-PCR otherwise K562 cell lines. Additionally we used as positive control a patient with Gorlin syndrome. Afterwards we measured Ptch1 together with BCR-ABL levels during the follow-up emphasizing that the most patients with high level of Ptch1 at diagnosis developed an Abl KD mutation (8/10). *Conclusions.* We showed for the first time a reciprocal correlation between BCR-ABL levels and activation of Hedgehog signalling in CML. We suggest that the aberrant Hh pathway activation is a feature of patients with CML Sokal high risk and a striking link with the development of Abl KD mutation is showed. Finally we suggest that Pcth1 could represent a surrogate marker for the molecular monitoring and a novel target to support classical TKIs treatment.

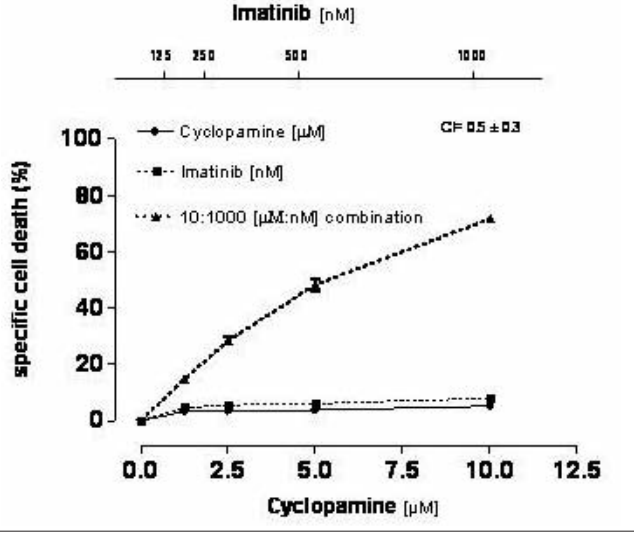

Figure 1. Synergism STI and cyclopamine.

0128

TWIST-1, A NEW PROGNOSTIC FACTOR IN CHRONIC MYELOID LEUKEMIA

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Chronic Myeloid Leukemia (CML) is one of the better characterized models for studying the properties of cancer stem cells and resistance to tyrosine kinase inhibitors. A significant proportion of CML patients develop resistance to these agents, and in 30% of cases, the mechanism of resistance is unknown. Analysis by Affymetrix chip of the sensitive and resistant forms of the CML cell line, KCL22, showed that the oncogene TWIST-1 is deregulated in Imatinib resistant cell line. Therefore, we studied the expression of Twist-1 and its involvement in the resistance of leukemic cells in samples from CML patients. For this study, we used primary blood cells from samples of healthy donors and CML patients at diagnosis, during follow-up of the disease and in the phase of relapse. Informed consent for the use of these cells for research was obtained in accordance with the Declaration of Helsinki and with approval from the Hospice Civils de Lyon ethic committee. Furthermore, we used cell lines, KCL22 and LAMA84 established from CML, for which we have sensitive and resistant clones to be cultured with 1 µM of Imatinib. We analyzed the expression of Twist-1 by qPCR in the two cell populations $CD34^+$ and $CD34^-$ obtained by CD34 immunomagnetic separation from patients with CML. Our results showed an overexpression of TWIST-1 during the progression of CML and in resistance to Imatinib. The analysis of 26 patient samples at diagnosis showed an overexpression of Twist-1 in 9 resistant patients to 9 including 4 patients that developed cytogenetic resistance without mutation or overexpression of BCR-ABL in the 12 months following their treatment. Furthermore, we observed by qPCR and Western blot analysis that TWIST-1 is overexpressed in Imatinib resistant clones of KCL22 and LAMA84 cell lines as compared to their sensitive counterpart. Finally, the use of siRNA specific for Twist-1 in resistant LAMA84 cells partially restored their sensitivity to Imatinib as shown by a decrease in the number of cells and viability in the presence of Imatinib. These results suggest a direct involvement of Twist-1 resistance in Chronic Myeloid Leukemia in the presence of Imatinib. Our results demonstrate for the first time that Twist-1 is a molecular marker to identify, at diagnosis, potentially resistant CML and monitor their response to treatment, even when the resistance mechanism is unknown.

0129

THE AURORA-KINASE INHIBITOR AS703569 EXERTS GROWTH-INHIBITORY AND APOPTOSIS-INDUCING EFFECTS IN PRIMARY CML CELLS AND CELL LINES EXPRESSING VARIOUS IMATINIB-RESISTANT BCR/ABL MUTANTS

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Imatinib resistance is a major clinical problem and challenge in chronic myeloid leukemia (CML). In most patients, drug-resistant mutants of BCR/ABL are detectable. Although several of these mutants still are responsive to second generation BCR/ABL kinase inhibitors such as nilotinib or dasatinib, drug responses are often short-lived. In addition, the BCR/ABL mutant T315I confers resistance against all conventional second-generation BCR/ABL inhibitors, including nilotinib, dasatinib, and bosutinib. More recent data suggest that several Aurora kinase inhibitors (AuK) block BCR/ABL T315I. We examined the growth-inhibitory effects of the AuK AS703569 (Merck-Serono, Darmstadt, Germany) on primary CML cells (chronic phase, n=4), the CML cell line K562, and Ba/F3 cells transfected with various drug-resistant mutants of BCR/ABL. As assessed by 3H-thymidine-uptake, AS703569 was found to inhibit the proliferation in imatinib-sensitive and imatinib-resistant K562 cells, in primary CML cells in all donors tested (n=4) as well as in Ba/F3 cells harbouring various imatinib-resistant mutants of BCR/ABL (E255K, Y253F, H396P, T315I). The effects of AS703569 on BCR/ABL-transformed cells were dose-dependent with IC50 values ranging between 10-100 nM in K562 cells, 10-1000 nM in primary CML cells, and 1-100 nM in Ba/F3 cells exhibiting BCR/ABL mutants.

Growth-inhibitory effects of AS703569 on CML cells were accompanied by signs of DNA endoreduplication and signs of apoptosis suggesting the involvement of multiple mechanisms and drug actions. To confirm drug effects on BCR/ABL, Western blot experiments using anti-pCrkL were performed and revealed that AS703569 blocks BCR/ABL activity at 1 μM in both K562 cells and Ba/F3 cells harbouring BCR/ABL T315I. In addition, AS703569 was found to block Aurora kinase A phosphorylation in K562 cells. In summary, our data show that the novel AuK AS703569 produces growth inhibition and apoptosis in BCR/ABL-transformed cells including those harbouring imatinib-resistant BCR/ABL mutants. Whether AS703569 also produces anti-CML effects in patients with advanced CML remains at present unknown.

0130

HEME OXYGENASE 1 (HO-1) MAY HAVE A ROLE IN RESISTANCE OF CML CELLS TO FIRST AND SECOND GENERATION TKIS

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Background. The advent of imatinib, an inhibitor targeted specifically for BCR-ABL, represented a significant medical advance in Chronic myeloid leukemia (CML) therapy. However, some patients become resistant or intolerant to treatment and second generation tyrosin kinase inhibitors (TKIs) may overcome these problems. Heme oxygenase (HO) is a rate-limiting enzyme in heme degradation (from hemoproteins and hemoglobin), leading to the generation of free iron, biliverdin and carbon monoxide. HO-1 expression has been reported as an important protective endogenous mechanism against physical, chemical and biological stress. It has been recently showed that the BCR/ABL oncoprotein promotes expression of HO-1 in CML cells and an over expression of HO-1 is able to inhibit imatinib induced apoptosis. We therefore investigated if an increased HO-1 activity is able to make cells resistant to second generation TKIs such as nilotinib and dasatinib. *Methods.* K562 cells were incubated for 24 hrs with imatinib 1 uM, or dasatinib 2 nM, or nilotinib 100 nM alone, or with an inductor of HO-1 (Hemin 50 uM, HE, or Cobalt protoporphyrin, CoPP, 10 uM) or the combination of both. After drug treatment, the viability of cells was evaluated by the ATP-lite1step assay (PerkinElmer). Gene expression of HO-1, HO-2 (the non-inducible form of heme oxygenase), and Nrf2 (Nuclear factor erythroid-derived 2) (a protein that regulates many factors involved in the oxidative stress, including HO-1) was assessed by Real time PCR (Applied Biosystems, 7900 Fast Real Time PCR). In addition, we evaluated stress oxidative production by measuring the ROS formation. The results are expressed as mean±S.E.M. and the statistical analysis was performed using student's t test. A value of $P<0.05$ was considered as significant. *Results.* We found that HO-1 gene expression was increased about 300 fold after HE or CoPP treatment either alone or in the presence of IM, while the expression of Nrf2 and HO-2 was not changed. The addition of HE or CoPP was able to overcome the inhibitory effect of IM (1 uM) on K562 cells ($P<0.002$) while the cytotoxic effect of IM was restored by adding an inhibitor of HO-1 (Tin-mesoporphyrin, SnMP, 10 uM) to the combination ($P<0.002$). Almost identical results were obtained with dasatinib and nilotinib ($P<0.002$). Moreover, IM 1uM was able to increase ROS formation, and this effect was inhibit by HE or CoPP and restored in presence of SnMP. *Conclusions.* In conclusion, we confirm that HO-1 may represent a mechanism of resistance to IM and we showed that the same mechanism may apply to the other TKI (dasatinib and nilotinib). It remains to better define the role of ROS in this mechanism of resistance.

0131

THE TUMOUR SUPPRESSOR PTEN: DIFFERENTIAL EXPRESSION PATTERNS IN CHRONIC MYELOID LEUKAEMIA AND THE ROLE OF PROMOTER METHYLATION

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Background. Chronic Myeloid Leukaemia (CML) is a broadly tri-phasic myeloproliferative neoplasm initiated by the BCR-ABL1 fusion oncogene and resultant fusion protein. Disease progression from an indolent chronic phase (CP) via an intermediate accelerated phase (AP) to an aggressive blast crisis (BC) is often characterised by the accrual of multiple genetic abnormalities, although the mechanism driving this process is poorly understood. PTEN (Phosphatase and Tensin Homolog) is a tumour suppressor gene, whose protein product acts as a phosphatase antagonist of PI3K/AKT and its down-regulation or loss has been described in several types of malignancies and is linked to disease progression. *Aims.* The aims of this project were to investigate PTEN expression in CML and to ascertain whether promoter CpG hypermethylation was a factor in its down-regulation. *Methods.* Gene expression of and PTEN was determined using real-time quantitative PCR (RT-qPCR) in a total of 75 patient samples in varying stages of CML and 23 normal blood samples. Differences in expression between normal, diagnostic, BC and remission samples were evaluated using the Kruskal-Wallis with Dunn's Post Test. Protein levels were determined in 10 CML patients (6 Diagnostic, 1 AP, 3 Remission) through flow cytometry. PTEN promoter hypermethylation was investigated by performing methylation-specific PCR (MSP) and methylation-sensitive restriction assays (MSR) targeting previously characterised CpG islands in the intronic region 5' to exon 1. The PCR products were run on 2% agarose gels. *Results.* RT-qPCR data showed that PTEN was significantly ($P<0.001$) down-regulated at presentation and AP/BC compared with normal and remission levels. Mean protein expression levels, as measured by flow cytometry and expressed as a ratio against the background fluorescence maxima, were 3.08, 4.72, and 12.70 for the diagnostic, AC and remission, respectively. Neither the MSP nor the MSR assay showed any significant CpG methylation upon gel visualisation. *Conclusions.* PTEN expression is significantly down-regulated in both diagnostic and BC CML samples when compared to normal and remission samples, and this was also seen at the protein level. Results from the MSP and MSR assays for promoter hypermethylation suggest that CpG methylation is not significantly involved in the down-regulation of PTEN in CML.

Chronic myeloid leukemia - Clinical 1

0132

LONG TERM OUTCOME (PROGRESSION-FREE SURVIVAL) IN PATIENTS RECEIVING NILOTINIB FOR IMATINIB FAILURE WHO MEET SUBOPTIMAL RESPONSE CRITERIA ACCORDING TO EUROPEANLEUKEMIA NET 2009 CML RECOMMENDATIONS

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Background. The EuropeanLeukemia Net (ELN) 2009 recommendations for the treatment of patients with newly diagnosed Philadelphia chromosome positive (Ph+) chronic myeloid leukemia in chronic phase (CML-CP) with imatinib is an important tool for physicians and now includes provisional recommendations for treatment milestones with nilotinib and dasatinib for patients with CML-CP who fail imatinib (Baccarani et al, J Clin Oncol. Dec 2009). *Aims.* In this landmark analysis, the response of patients with imatinib-resistant or -intolerant Ph+ CML-CP (N = 321) treated with nilotinib in the 2101 nilotinib phase 2 study was evaluated based on 2009 ELN suboptimal response criteria for second-line TKIs. *Methods.* Patients were grouped into 3 cohorts based on ELN 2009 recommendations: better than suboptimal response, suboptimal response, or treatment failure (and warnings). A landmark analysis of these 3 cohorts was conducted at 3-, 6-, and 12-month milestones from the initiation of nilotinib to determine the progression-free survival (PFS) of patients at 12 and 24 months. Patients who progressed or died prior to the landmark assessment date were excluded from the analysis at each given time point. *Results.* At 3 months, the majority of patients on nilotinib (57%) had a response better than ELN suboptimal response and achieved a PFS of 93% at 12 months and 79% at 24 months. Only a few patients (6%) were considered ELN suboptimal responders at 3 months and these patients had a PFS rate of 76% and 59% at 12 and 24 months, respectively. Those with early treatment failure had a PFS rate of 70% at 12 months and 41% at 24 months. At 6 months, similar trends were noted for all 3 patient cohorts (Table). The largest cohort of suboptimal responders occurred using the 12-month criteria (n=70). At the 12-month landmark, patients with better than suboptimal response and suboptimal response were comparable in terms of 24-month PFS rates (P=.1227) while patients with treatment failure had the poorest PFS and this was significantly lower (P<.0001) (Table). *Conclusions.* This analysis demonstrates the 24-month PFS for patients with suboptimal response to second-line nilotinib at 12 months was similar to patients who had better than suboptimal response. These results suggest that the 2009 ELN provisional criteria for suboptimal response to second generation TKIs needs refinement as the designation of suboptimal response does not appear to predict for worse prognosis at all treatment milestones.

Table.

	n	PFS at 12 months	PFS at 24 months
3-month milestone			
Better than suboptimal	125	93%	79%
Suboptimal	13	76%	59%
Treatment failure	81	71%	41%
6-month milestone			
Better than suboptimal	86	100%	86%
Suboptimal	29	89%	71%
Treatment failure	91	82%	51%
12-month milestone			
Better than suboptimal	47	NA	94%
Suboptimal	70	NA	87%
Treatment failure	63	NA	46%

0133

HIGH DOSE IMATINIB INDUCTION (800 MG/DAY, 6 MONTHS) INDUCES SIGNIFICANTLY HIGHER CYTOGENETIC AND MOLECULAR RESPONSES IN PRE-TREATED CHRONIC PHASE CML -PHASE III CELSG 'ISTAHIT' TRIAL - FIRST RESULTS

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Background. Imatinib 400 mg/day represents the current standard treatment for patients with chronic phase (CP) CML. Recently presented randomized phase III trials revealed conflicting results concerning the more potent efficacy of high dose imatinib. *Design and Methods.* We present the first results from the final analysis of the multicenter, randomised, 2-arm phase III CELSG CML 11 "ISTAHIT"trial evaluating imatinib high dose (HD) induction (800 mg/day for 6 months) followed by 400 mg/day as maintenance (experimental arm B) compared to continuous imatinib standard dose (arm A) in pre-treated CP CML patients. All patients have finished a 12 months period of detailed documentation followed by a 12 months period of treatment continuation and/or follow up. ClinicalTrials.gov Identifier: NCT0032726). *Results.* 113 patients were randomized into arm A and 114 patients into the experimental arm B. No significant differences between treatment groups were observed regarding sex (44.5% male, 55.5% female), age (median: 46 years for both groups), and different pre-treatments, which included hydroxyurea (96%), interferon (72%), busulfan (17%) and "others"(26%; mainly AraC±other drugs). In contrast to complete hematological responses, major cytogenetic responses (MCyR) were significantly higher at 3 and 6 months in the HD arm B (month 3: 25.8% arm A, 48.3% arm B, P=0.002; month 6: 41.9% arm A, 58.8% arm B, P=0.029). Higher MCyR rates were also detected in the HD arm B during the imatinib maintenance phase from month 7 to 24 (month 12, i.e. the primary endpoint: 56.8% arm A, 64.4% arm B; month 24: 71.3% arm A, 73.9% arm B), but did not reach statistical significance. Of note, the effect of HD imatinib was even more pronounced on complete cytogenetic response (CCyR) rates with significantly improved CCyR rates during imatinib HD therapy (month 3: 7.5% arm A, 29.9% arm B, P<0.001; month 6: 20.4% arm A, 47.4% arm B, P<0.001) and thereafter (month 12: 31.8% arm A, 52.9% arm B, P=0.006). In line with these findings, major molecular response (MMRIS) rates were also significantly better at 3, 6 and even at 24 months in the HD arm B (month 3: 4.5% arm A, 15.7% arm B, P=0.007; month 6: 10.3% arm A, 34.3% arm B, P<0.001; month 24: 27.4% arm A, 43.2% arm B, P=0.036). In contrast to comparable non-haematological toxicities during the first 6 months of therapy, grade 3/4 haematological toxicities were significantly more common in the imatinib HD arm B (anaemia 1% arm A, 14% arm B, P<0.001; leukopenia/neutropenia 21% arm A, 35% arm B, P=0.033; thrombocytopenia 12% arm A, 30% arm B, P=0.001). *Conclusions.* Although the primary endpoint of the study (i.e. the achievement of a MCyR at 12 months) was not reached, this first randomized phase III trial in pre-treated CP-CML patients supports the concept of more rapid and higher rates of cytogenetic and molecular remissions with higher doses of imatinib. Moreover, this trial confirms the safety as well as the remarkable therapeutic efficacy of imatinib in heavily pretreated CP CML with MCyR rates of >70% and CCyR rates of ≥50%.

0134

LONG TERM OUTCOME OF 559 PH+ CHRONIC MYELOID LEUKEMIA PATIENTS TREATED FRONT-LINE WITH IMATINIB: 5-YEAR RESULTS OF 3 INDEPENDENT STUDIES OF THE GIMEMA CML WORKING PARTY

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Background. Imatinib (IM) is the golden standard for the front-line treatment of chronic myeloid leukaemia (CML). Our knowledge on the long term outcome of CML patients treated with IM in early chronic phase (ECP) is mostly based on the IRIS trial, a company-sponsored study. Only few other independent reports, based on relatively smaller monocentric experiences and/or with a short follow-up, have been published. *Aims.* To provide an independent evaluation of the treatment responses and the long-term outcome of ECP Ph+ CML patients treated with imatinib mesylate in a large national multicentric experience. *Methods.* Between January 2004 and April 2007, 559 patients were enrolled in an observational study and in 2 independent interventional studies of the GIMEMA CML WP (Clin Trials Gov. NCT00514488 and NCT00510926). Response monitoring was based on conventional cytogenetic examination of bone marrow cell metaphases every 6 months and RT Q-PCR evaluations of blood cells after 3, 6, 12 months, and every 6 months thereafter. Definitions: major molecular response (MMR): BCR-ABL/ABL ratio <0.1% IS; failures (according to European LeukemiaNet criteria): no complete hematologic response (CHR) at 6 months, no cytogenetic response (CgR) at 6 months, no partial CgR at 1 year, no complete CgR (CCgR) at 18 months, loss CHR or CCgR, progression or death; events: failure or treatment discontinuation for any reason. All the calculations have been made according to the intention-to-treat principle. *Results.* 559 patients were treated with 400 mg (76%) or 800 mg (24%) IM daily. The median follow-up is currently 54 months. Either responses and outcome, overall and by Sokal score, are detailed in Table 1. Considering all the patients, the CCgR rate was 79% at 12 months, and 88% overall; the MMR rate was 54% at 12 months, and 83% overall. The 5-year event-free survival, failure-free survival and overall survival were 71%, 81% and 91%. The responses of low and intermediate risk patients were not significantly different (data not shown). All responses of high risk patient were significantly inferior. *Conclusions.* Our data confirms the results of IM treatment in a large, nationwide, multicentric experience, and point out that better therapeutic strategies should be developed for high risk patients.

Table 1.

	Total	Low Risk	Int. Risk	High Risk	p value
Patients, n (%)	559	219 (39)	216 (39)	124 (22)	/
Age, y, median (range)	52 (18-84)	44 (18-69)	61 (18-84)	52 (21-79)	/
CCgR at 12 months, %	79	85	83	65	< 0.01
MMR at 12 months, %	54	62	58	44	< 0.01
CCgR overall, %	88	95	94	79	0.11
MMR overall, %	83	92	89	72	< 0.01
EFS (5-year), %	71	79	74	50	< 0.01
FFS (5-year), %	81	89	83	56	< 0.01
OS (5-year), %	91	96	91	79	0.01

0135

EARLY MOLECULAR RESPONSE TO NILOTINIB IN PATIENTS WHO FAILED IMATINIB IS ASSOCIATED WITH A HIGHER PROBABILITY OF CYTOGENETIC RESPONSE IN CHRONIC MYELOID LEUKEMIA (CML)

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Background. Nilotinib is a potent and selective inhibitor of the BCR-ABL kinase, approved for the treatment of chronic phase (CML-CP) or accelerated phase (CML-AP) Philadelphia chromosome positive (Ph+) CML in adult patients resistant to or intolerant of prior therapy, including imatinib. Patients treated with nilotinib for imatinib failure may experience different response dynamics than those treated with imatinib in the frontline setting. Many patients treated with nilotinib in the second line achieved early cytogenetic responses with a median time to MCyR and CCyR of 2.8 months and 3.3 months, respectively. *Aims.* A previous landmark analysis demonstrated the predictive value of molecular response at 3 months on nilotinib for the achievement of subsequent cytogenetic response (Branford S, *et al.* Blood 2009;114:1275-6). Here, we investigated whether early molecular response before 3 months was associated with subsequent cytogenetic response. *Methods.* Imatinib resistant or intolerant patients with CML-CP (N=321) enrolled on the phase 2 registration trial of nilotinib were grouped by 1-month BCR-ABL transcript levels. Landmark analyses were performed to evaluate the probability of cytogenetic response with a minimum of 24 months follow-up. *Results.* Overall, 19% of patients achieved molecular response of BCR-ABL (IS) ≤ 10% within 1 month of starting nilotinib. Patients with BCR-ABL (IS) >1% - ≤10% (n=37) had a higher probability of achieving MCyR by 12 months (84%) compared with patients with BCR-ABL (IS) >10% (55%) (n= 202; P<.0001). The probability of achieving CCyR by 12 months was also higher in patients with BCR-ABL (IS) >1% -≤10% compared with patients with BCR-ABL (IS) >10% (72% vs. 38%; P<.0001). Similar trends in the probability of achieving MCyR and CCyR on nilotinib by 12 months were observed for patients when analyzed by the presence or absence of baseline mutations (Table). Similar results were also seen when analyzing the probability of cytogenetic response by 24 months. *Conclusions.* Molecular response to nilotinib at 1 month was associated with cytogenetic responses by 12 months, even in the presence of baseline mutations. While treatment decisions based on 1 month BCR-ABL transcript levels are not recommended, these data indicate that reductions in BCR-ABL transcripts may occur before 3 months on nilotinib and were associated with subsequent cytogenetic responses in these patients.

Table. Estimated MCyR and CCyR by 12 months by BCR-ABL levels at 1 month on nilotinib therapy.

	BCR-ABL (IS) at 1 Mo (%)		
	> 1 – ≤ 10	> 10	P-value (log-rank test)
MCyR by 12 Months			
All pts, n = 239	84%	55%	< .0001
Pts with baseline mutations, n = 95	76%	42%	< .0001
Pts without baseline mutations, n = 121	89%	64%	< .0001
CCyR by 12 Months			
All pts, n = 241	72%	38%	< .0001
Pts with baseline mutations, n = 96	58%	25%	.0022
Pts without baseline mutations, n = 122	89%	45%	< .0001

0136

ONLY A HIGH RELATIVE RISK (SOKAL OR EURO) PREDICTS FOR RESPONSE TO IMATINIB. AN EUROPEAN LEUKEMIANET EUTOS STUDY

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The EUTOS (European Treatment and Outcome Study) CML Registry dataset contains the data of 1955 patients who were enrolled in prospective studies of treatment with Imatinib (IM) or IM-containing regimes in France (n = 526), Germany and Switzerland (n = 657), Italy (n = 513), the Nordic Countries (Denmark, Finland, Norway, Sweden, n = 140), and the Netherlands (n = 119). Eligibility criteria were diagnosis of chronic phase Ph^+ or $BCR\text{-}ABL^+$ CML between 2002 and 2006, age ≥18 years, and start with any kind of IM-based treatment in a prospective study within 6 months from diagnosis. The data of these patients were analyzed to identify the baseline and the response-related factors which are associated with outcome. The baseline characteristics that have been associated with response and outcome in all CML studies are represented by two risk scores (Sokal *et al.*, Blood 1984;63:789-799; Hasford *et al.*, JNCI 1998;90:850-858) including age, spleen size, platelet count and the percent of blood myeloblasts, eosinophils and basophils, prior to any treatment. These risk scores were based on data of patients treated with conventional chemotherapy and with Interferon-α based regimes, respectively. In the pre-IM era, both risk classifications identified three risk groups, low, intermediate and high. The Sokal Score's cutpoints correspond to a relative risk (RR) and lie at <0.8, 0.8-1.2 and >1.2. The Euro Score's cutpoints lie at <781, 781-1480, and >1480. After the introduction of IM, both risk classifications were already reported to be of some utility (reviewed in Baccarani et al, Blood 2006; 108: 1809-20 and JCO 2009;27:6041-6051) but so far they were not specifically analysed and validated for prognostic value. Since the best early surrogate marker of late outcome is the complete cytogenetic response (CCgR) and since the lack of a CCgR at 18 months is identified as a "failure"in IM-treated patients (Baccarani *et al.*, JCO 2009;27:6041-6051), we analysed the relationship between risk class and CgR at 18 months. The analysis was based on 728 (Sokal score) and 727 (Euro score) patients in whom the information on the CgR response at 18±2 months was available. We have calculated also the cumulative incidence of CCgR at 3 years, based on all patients where the risk could be determined (1710 for Sokal, 1707 for Euro). The data are reported in Table 1, showing that although the overall difference was significant for both scores, the most important difference was between high risk patients and low, or low + intermediate risk patients.

Table 1. Result: sokal and euro score versus CCgR.

		LOW		INTERMEDIATE		HIGH
Sokal.	No. (%)	667 (39%)		628 (37%)		415 (24%)
Sokal.	Cumulative incidence of CCgR at 3 years, %	95%	°	93%	°	82%
Sokal.	No. (%) with a Cg test at 18 months	303		279		146
Sokal.	No. (%) in CCgR at 18 months	281 (93%)	*	251 (90%)	*	115 (78%)
Hasford.	No. (%)	655 (38%)		860 (50%)		192 (11%)
Hasford.	Cumulative incidence of CCgR at 3 years, %	93%	°	91%	°	79%
Hasford.	No. (%) with a Cg test at 18 months	295		373		59
Hasford.	No. (%) in CCgR at 18 months	274 (93%)	+	323 (87%)	+	49 (83%)

° p-values: < 0.001 for Low + Intermediate vs. High (over the whole observation time, Gray test)

* p-values: Low vs. Intermediate 0.23; Intermediate vs. High 0.002; Low vs. High < 0.001 (Chi^2-Test)

+ p-values: Low vs. Intermediate 0.01; Intermediate vs. High 0.46; Low vs. High 0.02 (Chi^2-Test)

In conclusion, in IM-treated patients both scores were validated, with particular emphasis on the high risk. These data support the European LeukemiaNet recommendations identifying a high risk score as a baseline warning factor (Baccarani *et al.*, JCO 2009;27:6041-6051), and emphasize the importance of collecting and reporting sound clinical data before any treatment, so as to make it possible to calculate the risk. However both scores are under revision, in order to improve their prognostic value and to investigate which other prognostic factors can be of help in the tyrosine kinase inhibitors era.

0137

ACTIVE DOSE RE-ESCALATION OF NILOTINIB FOLLOWING DOSE REDUCTION IS ASSOCIATED WITH IMPROVED EFFICACY IN PATIENTS WITH CHRONIC PHASE CHRONIC MYELOID LEUKEMIA (CML-CP) WHO FAIL IMATINIB

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Background. Nilotinib is approved for the treatment of CML-CP in adult patients resistant to or intolerant of prior therapy, including imatinib. Overall, nilotinib is generally well tolerated with minimal cross-intolerance to imatinib and limited off-target effects. In the Expanding Nilotinib Access in Clinical Trials (ENACT) study, the duration of grade 3/4 study-drug related AEs was typically short (1-3 weeks) with the onset of new such AEs decreasing over time. Despite this, more than 50% of eligible patients did not undergo re-escalation to the optimal dose of 400 mg BID. *Aims.* The purpose of this analysis was to study the impact of nilotinib dose re-escalation on responses in the ENACT study. *Methods.* We analyzed patients with CML-CP from ENACT who failed prior imatinib, initiated nilotinib at 400 mg BID, and had ≥ 1 nilotinib dose reduction (N=999). Dose re-escalation was recommended but not mandated, as per protocol. Analysis of the correlation of dose re-escalation with cytogenetic response was performed on all available CML-CP patients from participating ENACT sites. The French subset of patients from ENACT was also analyzed separately to assess patients who are being monitored for molecular response according to ELN recommendations. The starting dose was 400 mg BID with 400 mg QD used for dose reduction due to AEs. Re-escalation was permitted when AEs ≤ to Grade 1. *Results.* Of 1,376 patients with CML-CP who failed prior imatinib and initiated nilotinib 400 mg BID (59% resistant, 40% intolerant, 1% both), 999 (77%) had ≥1 nilotinib dose reduction; 444/999 (44%) attempted re-escalation to 400 mg BID and 385/444 (87%) successfully re-escalated (for ≥28 days without dose interruption of >5 days). In patients who successfully re-escalated, subsequent median dose intensity was high at 796 mg/day (near planned dose) and was maintained for a median of 220 days. Patients who successfully re-escalated to nilotinib 400 mg BID experienced higher cytogenetic responses than patients who did not attempt re-escalation (n=555) or in whom successful re-escalation was not possible (n=59). Overall, major cytogenetic response (MCyR) was achieved in 52% of patients who had successful re-escalation and in 43% who did not. Within the French subset (n = 165), 125 (76%) had ≥1 dose reduction; 41/125 (33%) attempted a re-escalation to 400 mg BID and 36/41 (88%) were successfully re-escalated. Overall, MCyR was achieved in 75% of patients who had a successful re-escalation to 400 mg BID and in 53% who did not. Major molecular response (MMR) was achieved in 47% of patients who re-escalated and in 44% who did not. *Conclusions.* Active re-escalation of nilotinib following dose reduction results in an increased probability of cytogenetic and molecular response in patients with CML-CP, especially when combined with regular monitoring. The use of the approved nilotinib doses (400 mg BID starting dose and 400 mg QD reduced dose) is associated with improved patient response rates when re-escalation is used appropriately.

0138

HIGH BCR-ABL EXPRESSION LEVELS AT DIAGNOSIS MAY PREDICT UNFAVOURABLE CML RESPONSES TO IMATINIB THERAPY

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Background and Aims. The advent of BCR-ABL tyrosine kinase inhibitors (TKIs) such as imatinib mesylate (IM) dasatinib and nilotinib (NIL) has dramatically changed the natural history of Chronic Myeloid Leukemia (CML). However, it is still unclear if: i) BCR-ABL transcript variants (i.e. e13a2, e14a2) predict response to IM; ii) high levels of BCR-ABL transcripts at diagnosis are indicative of an aggressive leukemic clone displaying increased genomic instability. *Patients and Methods.* To address these issues we assessed BCR-ABL transcripts in 135 patients with chronic phase (CP) CML accrued at our Institution between January 2003 and June 2009. All patients received IM therapy at 400 mg daily with the exception of ten who were enrolled in an investigational trial and received NIL-therapy at 800 mg daily. Median follow-up was 27 months (range 3-54). Hematological, cytogenetic and molecular responses were rated according to the European Leukemia Net (ELN 2006) guidelines. Peripheral blood samples were used for BCR-ABL determination by quantitative real-time polymerase chain reaction, following the suggested recommendations and according to the International standardized Scale (IS). *Results.* CP-CML patients included in this series were initially stratified according to BCR-ABL transcript variants and analyzed for their main clinical, cytogenetic and molecular characteristics. Age, sex, hemoglobin, white blood cells (WBC), platelets counts and Sokal score did not differ between the two populations. We also found no statistical difference between the two CML groups when considering rates of complete cytogenetic remission after 12 months of treatment (P=1.0). The only significant difference between the two subgroups was the amount of BCR-ABL expression at diagnosis, with e13a2 individuals displaying much higher levels of BCR-ABL as compared to those with the e14a2 variant (P<0.0001). We next clustered all subjects in optimal responders (ORs) and suboptimal/resistant (S/R) patients according to the 2006 ELN criteria and correlated response to therapy with different clinical and molecular characteristics. We found that only the amount of BCR-ABL transcripts at diagnosis predicted response to IM, with an increased number of S/R patients in the group expressing higher levels of BCR-ABL. Indeed, the median amount of BCR-ABLIS transcript at diagnosis displayed by patients that failed TKI therapy or achieved a suboptimal response was significantly higher (106.68IS) than that of patients obtaining an optimal response (67.2IS; P=0.01). As WBC counts were not significantly different between ORs and S/R patients (P=0.5), increased amounts of BCR-ABL transcripts are probably representative of the aggressiveness of the leukemic clone that, in turn, might identify CML patients at higher risk of progression. *Conclusions.* In summary, our findings support the notion that high levels of BCR-ABL expression at the time of diagnosis may identify CML patients less likely to benefit from IM therapy.

0139

RAPID INITIAL DECLINE IN BCR-ABL LEVELS IS ASSOCIATED WITH SUPERIOR RESPONSES IN IMATINIB-RESISTANT OR -INTOLERANT CHRONIC MYELOID LEUKEMIA PATIENTS IN CHRONIC PHASE (CML-CP) TREATED WITH NILOTINIB

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Background. A mathematical model established using molecular data from patients with newly diagnosed CML-CP who were randomized to the imatinib arm of the International Randomized Study of Interferon and STI571 (IRIS) trial demonstrated that the majority of patients experienced a biphasic decline in BCR-ABL transcripts, with a rapid initial decline (α) followed by a steady long-term decline (β). *Aims.* Here we have applied this model to the molecular response data from patients with CML-CP with prior resistance or intolerance to imatinib treated with nilotinib 400mg bid. *Methods.* Patients from the nilotinib phase 2 registration study who had at least 24 months of follow-up and a sufficient number of PCR data points to support parameter estimation were included in the model (N=259). The time course of BCR-ABL transcript reduction (IS) was modeled as a biexponential function ($R(t) = Ae^{\alpha t} + Be^{\beta t}$). Patient parameters were estimated using nonlinear mixed effects modeling. The α parameter describes the initial decline in log10 (R) upon treatment start while β describes the shallower slope of the subsequent log10 (R) dynamics in patients. *Results.* As with patients treated with imatinib on IRIS, the patient population was well-described by this model. However, unlike in IRIS, the majority of patients displayed monophasic dynamics where only the α slope was observable. Also in contrast to IRIS, the α parameter showed a bimodal distribution (Fig 1b), with patients displaying 1 of 2 typical responses, a shallow α slope corresponding to a lesser initial decline in BCR-ABL transcripts (> -5/yr; n=165) or a steep α slope (< -5/yr; n = 94) (Figure 1a, 1c). A steep α slope (36% of pts) was associated with superior responses and event-free survival (EFS), with 24 month rates of complete cytogenetic response, major molecular response, and EFS of 94%, 77% and 83% for patients with α < -5/yr versus 25%, 6%, and 40% for patients with α > -5/yr (P<.0001 for all comparisons). Three PCR data points collected within months 3-6 of therapy could reliably estimate the α slope. The β parameter, generally observable only in patients with a steep α, was similar to that seen in IRIS, with a median yearly steady state reduction in log10 transcript levels of -0.66/yr (range, -3.5/yr-5.9/yr). Only 9 (3.6%) patients had $\beta > 0$ with 95% confidence, suggestive of molecular relapse. *Conclusions.* Mathematical modeling demonstrated that treatment with nilotinib can be described by 2 main parameters: an initial decline in BCR-ABL transcript levels (α) and a longer, more sustained decline (β). Two patterns emerged in the slope of the initial decline, steep (α < -5/yr) or shallow (α > -5/yr). A steep α was shown to be associated with superior response and EFS outcomes. This bimodal distribution of α, which could be estimated using PCR data collected during the first 3-6 months of therapy, may be an early predictive tool for longer-term outcomes of patients on second-line nilotinib who have failed prior imatinib therapy.

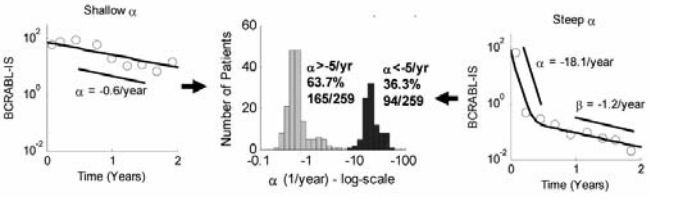

Figure 1. a) Typical shallow α patient; b) distribution of a slopes; c) typical step α patient.

0140

CORRELATION OF IMATINIB PHARMACOKINETICS WITH CLINICAL RESPONSE IN 314 PATIENTS WITH CHRONIC MYELOID LEUKEMIA - INTEGRATED DATA FROM SIX JAPANESE STUDIES -

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Background. Despite the outstanding results of imatinib (IM) for the treatment of chronic myeloid leukemia (CML), some patients show poor molecular response to IM therapy. A number of factors may underlie this variability, including BCR-ABL mutations, Sokal risk score at baseline, and pharmacokinetics-related interindividual variation affecting IM metabolism and/or drug transporters. Recently, it has been suggested by several large studies that variation in trough IM plasma levels can affect IM response among CML patients. However, contradictory data showing no correlation between trough IM plasma concentration and molecular response has also recently been reported. In

Japan, several groups independently evaluated IM pharmacokinetics in CML patients and the majority have not reported a significant correlation between trough IM plasma concentration and response. *Aims and Methods.* To further examine and clarify the potential correlation between steady state trough IM plasma concentration and clinical response in Japanese chronic-phase CML patients, we integrated data from six independent studies. To determine trough IM plasma concentrations, a central laboratory was utilized that employed high-performance liquid chromatography-tandem mass spectrometry. The study protocol was approved by the Ethics Committee and all patients gave written informed consent. *Results.* Three hundred fourteen Japanese patients with chronic-phase CML and a median age of 60 years (range; 16-91 years) were integrated in this analysis (male vs. female, 189 vs. 125). One hundred ninety (60.5%), 59 (18.8%), 48 (15.3%), and 17 (5.4%) patients were treated with 400 mg, 300 mg, <300 mg, and >400 mg of IM daily, respectively, and the median duration of IM therapy was 1435 days. The mean and median trough IM plasma concentrations were 1010.5 ng/mL and 900 ng/mL, respectively, and the distribution of trough IM plasma concentration at all doses is illustrated in Figure 1. Among the 190 patients treated with 400 mg daily, weak correlations between trough IM plasma concentration and age (r^2=0.033), body weight (r^2=0.027), and body surface area (r^2=0.04) were observed; however, the large interpatient variability in trough concentrations suggests that these associations are likely not clinically significant. When patients were divided into quartiles based on trough IM plasma concentration distribution, no correlations with baseline characteristics were detected. Notably, significant differences among the quartiles in the percentage of patients with a complete cytogenetic response (CCyR; P=0.008) and a major molecular response (MMR; P=0.007) were identified. In addition, trough IM plasma concentration at all doses was significantly higher among patients with a MMR than those without a MMR (P=0.0003). Among the patients treated with 400 mg, a significant difference in trough IM plasma concentration between those with a MMR than those without a MMR (P=0.023) was again detected. *Conclusions.* Higher trough IM plasma concentration was associated with the likelihood of achieving a CCyR or a MMR in our large Japanese CML patient cohort. Thus, in addition to BCR-ABL mutation analysis, our data indicate that clinical IM blood-level testing may improve the efficacy of IM therapy among CML patients.

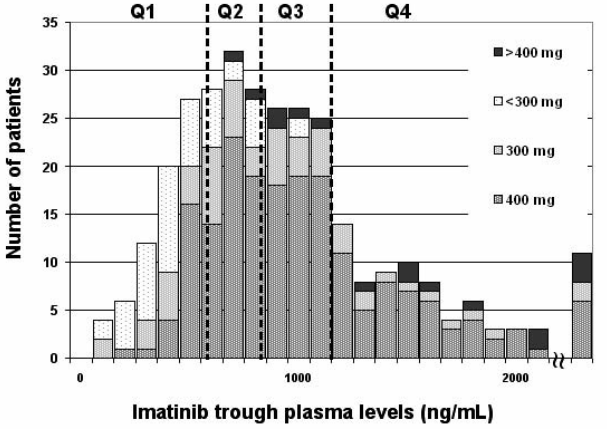

Figure 1. Distribution of IM Cmin at steady state (n=314).

0141

OCT-1 EXPRESSION LEVELS PREDICT MOLECULAR RESPONSES IN CHRONIC PHASE CML PATIENTS ON FIRST LINE IMATINIB TREATMENT

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Background. Most newly diagnosed chronic phase CML patients exhibit cytogenetic and molecular responses under imatinib therapy. However, resistance and suboptimal response occur in a cohort of patients. BCR-ABL kinase domain mutations and several BCR-ABL independent mechanisms, such as cytogenetic aberrations and clonal evolution are considered as leading causes of resistance and progression. White *et al*. have shown that imatinib efficacy depends on intracellular drug levels which are influenced by the activity of the influx human organic cationic transporter protein (OCT-1) and efflux transporter protein multidrug resistance 1 (MDR1) (White *et al.*, Blood 2006; Mahon *et al.*, Cancer Res 2008). *Aims.* We therefore analyzed the predictive role of MDR1 and OCT-1 expression levels of chronic phase CML patients on molecular responses during first line therapy with imatinib. *Patients and Methods.* A cohort of 133 newly diagnosed chronic phase CML patients (47 f, 86 m; median age 53 years, range 16-75) treated with imatinib 400 mg/day in the German CML Study IV were investigated. Median follow-up for achievement of MMR (BCR-ABL expression according to the international scale ≤0.1%) was 16 months (range 5-75). MDR1, OCT-1 and BCR-ABL mRNA expression levels were determined by quantitative reverse transcription PCR (qRT-PCR) using LightCycler™ technology, normalized against beta-glucuronidase (GUS) expression and standardized according to the international scale (IS). Log-rank tests were performed to compare the time to MMR. *Results.* Within 12, 18 or 24 months of imatinib therapy, 24%, 44% and 54% of patients achieved MMR. After 12, 18 or 24 months, patients with OCT-1/GUS ratios ≥0.69 (46%) achieved MMR in an estimated rate of 30%, 52% and 63%, whereas those with initial OCT-1/GUS ratios <0.69 (54%) showed MMR in 17%, 36% and 44%, respectively (12 months, P=0.068; 18 months, P=0.045; 24 months, P=0.015). No correlation between MDR1/GUS ratios and the achievement of MMR was found after 12, 18 or 24 months. *Conclusions.* Pre-treatment expression levels of OCT-1 appear to predict the achievement of MMR under imatinib therapy in chronic phase CML patients over a period of 2 years of therapy.

0142

BOSUTINIB FOR THIRD-LINE TREATMENT OF CHRONIC PHASE (CP) CHRONIC MYELOID LEUKEMIA (CML) FOLLOWING RESISTANCE OR INTOLERANCE TO IMATINIB (IM) AND DASATINIB (D)

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Background. Bosutinib (SKI-606) is an orally available, dual Src/Abl kinase inhibitor. *Aims.* A phase I-II study was conducted to evaluate safety and efficacy of bosutinib in patients (pts) with CP CML after failure of imatinib and dasatinib subsequently. *Methods.* After collection of informed consent, pts received bosutinib 500 mg orally, daily. *Results.* Of 90 CP pts with prior exposure to IM and D, 43% were male, median age was 57 (21-79) years, and time from CML diagnosis to start of bosutinib was 6.5 (0.6-18.3) years, with a median follow-up of 17 months (0.3-35). The most frequent treatment emergent adverse events (TEAEs) were gastrointestinal (diarrhea (81%), nausea (46%), vomiting (38%)). These effects were low-grade, early onset and usually subsided spontaneously within the first 4 weeks of treatment (median duration of 27 days). Grade 3/4 TEAEs occurring in ≥5% of pts were diarrhea (8%) and rash (7%). Grade 3/4 hematologic lab abnormalities included thrombocytopenia (20%), neutropenia (10%) and anemia (9%). Few non-hematologic Grade 3/4 lab abnormalities were observed, notably 1 pt (1%) with elevated amylase, 4 pts (4%) with elevated lipase, and no occurrence of hyperglycemia or QTc prolongation. One incidence of grade 3 pleural effusion was observed with a pt in the setting of concomitant pneumonia and a history of recurrent pleural effusions on D. Median daily dose was 492mg for I-treated/DAS-resistant, 383mg for I-treated/DAS-intolerant pts; 32 pts (36%) required dose-reductions and 20 pts (22%) discontinued permanently bosutinib for toxicity. Among 40 pts with baseline testing, 18 (45%) had a Bcr-Abl mutation. In evaluable pts with mutations, 56% complete hematologic response (CHR) and 27% major cytogenetic response (MCyR) were observed, for evaluable pts with no mutation, 89% CHR and 38% MCyR. In pts with F317L, CHR occurred in 2/3 evaluable pts and MCyR in 2/4 evaluable pts. Hematologic responses were also observed in patients with M244V, G250E, Y253F, including 2/3 evaluable pts with P-loop mutations achieving CHR. No clinical activity was detected in pts with T315I. *Summary/Conclusions.* Bosutinib has an acceptable toxicity profile in CP CML patients with resistance or intolerance to both IM and D, with primarily low-grade and transient gastrointestinal AEs. Bosutinib demon-

strated clinical activity in the third-line setting, with 39% of pts achieving MCyR, including 30% with CCyR.

Table 1.

	IM Resistant + D Resistant n (%)	IM Intolerant + D Resistant n (%)	IM Resistant + D Intolerant n (%)	IM Intolerant + D Intolerant n (%)	Overall Total n (%)
Hematologic response					
Evaluable*	22	1	13	11	47
Complete	15 (68)	1 (100)	10 (77)	9 (82)	35 (74)
Cytogenetic response					
Evaluable*	24	0	14	14	54
Major	9 (38)	n/a	6 (43)	6 (43)	21 (39)
Complete	5 (21)		5 (36)	6 (43)	16 (30)
Molecular response					
Evaluable*	20	0	19	16	56
Major	2 (10)	n/a	8 (42)	5 (31)	15 (27)
Complete	1 (5)		5 (26)	4 (25)	10 (18)

* Pts without CHR, CCyR or CMR at baseline, and with post-baseline assessment for respective response

0143

UPDATE OF CYTOGENETIC AND MOLECULAR RESPONSE IN 88 ELDERLY PH+ CHRONIC MYELOID LEUKEMIA PATIENTS ENROLLED IN THE PHASE II EXPLORATIVE STUDY OF INTERMITTENT IMATINIB (IM) TREATMENT (INTERIM)

D Russo,[1] G Rosti,[2] G Martinelli,[2] M Malagola,[1] S Mirto,[3] D Turri,[3] M Gobbi,[1] I Pierri,[1] U Vitolo,[1] P Pregno,[1] E Morra,[3] E Pungolino,[3] F Di Raimondo,[1] F Stagno,[1] R Foà,[4] G Alimena,[4] M Breccia,[4] F Nobile,[3] B Martino,[3] A Rambaldi,[3] T Intermesoli,[3] G Saglio,[5] G Rege Cambrin,[5] G Visani,[3] G Nicolini,[3] P De Fabritiis,[6] E Abruzzese,[6] R Fanin,[1] M Tiribelli,[1] P Galieni,[3] C Bigazzi,[3] V Liso,[1] G Specchia,[1] E Angelucci,[3] E Usala,[3] C Musolino,[1] S Russo,[1] G Gaidano,[1] M Lunghi,[1] F Lauria,[1] M Bocchia,[1] A. D'Emilio,[3] A. Bosi,[4] V. Sanitini,[4] G. Quarta,[3] M. Girasoli,[3] G. Fioritoni,[3] R. Di Lorenzo,[3] C. Colombi,[4] M. Fogli,[2] M. Amabile,[2] N. Testoni,[2] A. De Vivo,[2] M. Baccarani[2]

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Background. Elderly CML patients treated with Imatinib (IM) in early chronic phase (CP) have similar cytogenetic response and survival compared with younger patients, but they show a lower compliance to standard IM therapy (400 mg/day). *Aims.* The aim of the study is to investigate if a stable CCgR that has been achieved with standard (daily administration) IM therapy can be maintained with the same dose of IM given intermittently (INTERIM). *Methods.* This interim analysis was conducted in 88 elderly patients (≥65 years) with Ph+ chronic myeloid leukemia (Ph+ CML) and with stable complete cytogenetic response (CCgR), who were enrolled in the INTERIM study and who, at present, are evaluable after a median of 13 months from the start of the study (28th April 2008) (range: 2-20 months). Briefly, INTERIM consisted on the intermittent administration of IM at the same dose given at the time of enrollment, by the following schedule: 1 week on / 1 week off for the 1st month; 2 weeks on / 2 weeks off for the 2nd and 3rd month; 1 month on / 1 month off from the 4th month thereafter. The CgR status has been assessed by FISH on peripheral blood every 3 months and if FISH (% of Ph+ cells) increased more than 1% in two consecutive examinations, evaluation of marrow cells metaphases was performed to confirm the loss of CCgR and to check for additional cytogenetic abnormalities. Quantitative molecular assessment of BCR-ABL transcript by RQ-PCR on peripheral blood was also due every 3 months during the study and mutational analysis of ABL was performed in case of loss of CCgR. In cases of loss of CCgR INTERIM was stopped and standard therapy (daily administration) was resumed. After 12 months, the patients who are in continuous CCgR are advised to continue the intermittent study schedule and to be followed indefinitely. *Results.* Of the 88 patients, 71, 68, 53 and 30 completed the 3rd, 6th, 9th and 12th month, respectively. The preliminary results of the first 12 months are reported here. The distribution of patients according to FISH results are shown in the Figure 1. As reported, only few patients (from 1 to 16%) showed an increased number of Ph+ cells by FISH >1%, but all maintained a CCgR when checked by conventional cytogenetic. Concerning the molecular response, as showed in Figure 2, 93 to 84% of patients maintained a major molecular response MMR (<0.1) according to International Scale (IS). *Conclusions.* The preliminary results at 12 months do not show negative trends both for cytogenetic and molecular response. These data suggest that INTERIM may be a useful strategy to test the minimum effective dose of IM and it could be proposed as a new treatment schedule in elderly patients to maintain a stable CCgR previously achieved with standard IM therapy

Acknowledgments: This work was supported in part by CML-Leukemia Net.

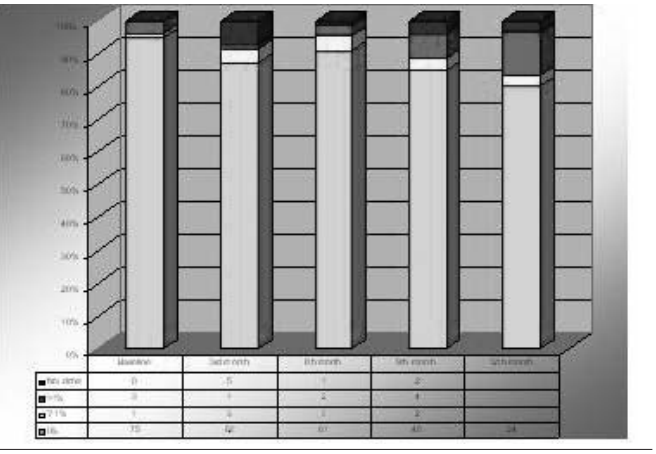

Figure 1. Distribution of patients according to FISH.

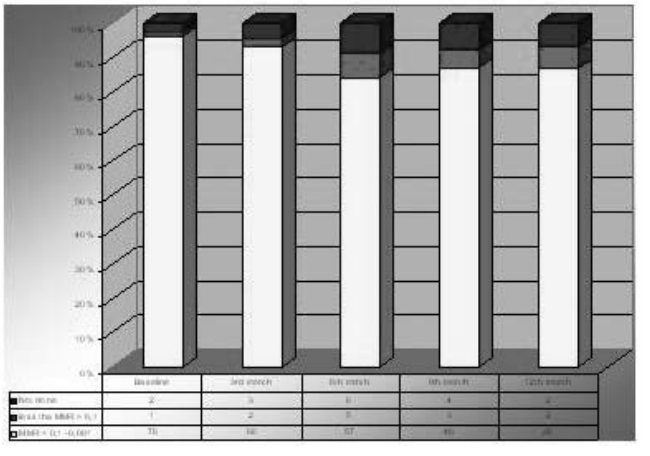

Figure 2. Distribution of patients according to BCR/ABL transcript leveles.

0144

THE PREDICTIVE IMPACT OF MDR1 EXPRESSION AND PRE-TUMOR BURDEN ON MOLECULAR, CYTOGENETIC AND CLINICAL OUTCOME OF CML PATIENTS ON NILOTINIB THERAPY AFTER IMATINIB FAILURE

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Background. Nilotinib has shown to be effective in case of resistance or intolerance to imatinib. Mutations in the BCR-ABL kinase domain and various BCR-ABL independent mechanisms, e.g. clonal evolution and pathways bypassing BCR-ABL are considered as leading causes of resistance. Besides, efficacy of imatinib and nilotinib depends on intracellular drug levels, which are influenced by the activity of the efflux transporter protein multidrug resistance 1 (MDR1). Cell proliferation data suggest overexpression of MDR1 as a cause of resistance to nilotinib (Mahon et al., Cancer Res 2008). *Aims.* We assessed the predictive impact of MDR1 expression levels and pre-treatment tumor burden of imatinib resistant CML patients on molecular and cytogenetic responses and progression-free survival (PFS) during second line therapy with nilotinib. *Methods.* Imatinib resistant patients in chronic phase CML treated with nilotinib (n=84) were investigated in a phase II study (AMN2101). Baseline BCR-ABL mutations were detected by D-HPLC and direct sequenc-

ing. MDR1 and BCR-ABL mRNA expression levels were determined by quantitative reverse transcription PCR (qRT-PCR) using LightCycler™ technology, normalized against beta-glucuronidase (GUS) expression and standardized according to the international scale (IS). Log-rank tests were performed to compare the time to major molecular response (MMR, BCR-ABL IS ≤0.1%), complete cytogenetic response (CCyR), and PFS. *Results.* Within 12 or 24 months of nilotinib therapy, 24% and 29% of patients achieved MMR. After 12 or 24 months, patients with MDR1/GUS ratios ≥2.0 (62%) achieved MMR in an estimated rate of 34%, whereas those with initial MDR1/GUS ratios <2.0 (38%) showed MMR in 13%, respectively (P=0.030). Further, BCR-ABL load prior to nilotinib revealed a significant impact on consecutive molecular response. BCR-ABL IS <28% separated best concerning prediction of MMR after 12 and 24 months (58% vs 20% and 58% vs 36%, P=0.0013). CCyR was attained in 52% and 57% of the patients with MDR1/GUS ratios ≥2.0 after 12 and 24 months, whereas those with MDR1/GUS ratios <2.0 showed CCyR in 25% and 35% (P=0.036). Moreover, a MDR1/GUS ratio at 2.0 also significantly dichotomized two groups for achievement of PFS: patients presenting MDR1/GUS ratios ≥2.0 reached PFS rates of 88% and 73%, whereas those with MDR1/GUS ratios <2.0 attained PFS in 71% and 50% after 12 and 24 months (P=0.036). *Conclusions.* Pre-treatment expression levels of MDR1 predict MMR, CCyR and PFS of imatinib resistant chronic phase CML patients within the first two years of treatment with nilotinib. These findings might allow risk stratification in order to tailor the individualized second line therapy in CML and should be validated prospectively.

0145

PRETREATMENT BCR-ABL TUMOR BURDEN AND MUTATION STATUS ARE INDEPENDENTLY PREDICTIVE FOR MOLECULAR RESPONSE IN CHRONIC PHASE CML PATIENTS TREATED WITH DASATINIB AFTER IMATINIB FAILURE

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Background. Major molecular response (MMR) is achieved in a substantial proportion of chronic phase chronic myeloid leukemia (CML-CP) patients treated with dasatinib after failure of imatinib therapy. However, recently published data suggest that the proportion is strongly dependent on the time of changing the TKI regimen. Since an early change has been shown to predict a better outcome, cutoff levels in the molecular scope are needed to determine the optimal time of dasatinib treatment initiation and to further specify prognostic groups. *Aims.* We sought to evaluate the prognostic significance of pretreatment BCR-ABL levels and BCR-ABL kinase domain mutation status for long term molecular outcome in CML-CP patients after failure of imatinib therapy. *Methods.* 135 CML-CP patients (72 male, 63 female, median age 61 years, range 22-77) with resistance to imatinib were included into the CA 180-013 phase II study (Bristol-Myers Squibb, Wallingford, CT) and received 70 mg dasatinib twice daily. 1,197 peripheral blood samples were analyzed by quantitative RT-PCR of BCR-ABL and beta-glucuronidase (GUS) and direct sequencing of the rearranged ABL kinase domain. The BCR-ABL/GUS ratio was determined according to the international scale (IS). *Results.* Median pretreatment BCR-ABL expression (IS) was 42.0% (range 3-215%), median BCR-ABL (IS) at the end of follow-up was 15.3% (range 0-214%) with a median follow-up of 21.9 months (range 0-35). BCR-ABL expression (IS) of less than 20% predicts a high cumulative incidence of achieving BCR-ABL (IS) levels of less than 1%, termed good molecular response (GMR). In the good risk group 15 of 25 pts (60.0%) achieved a GMR after a median of 8.3 months, in the poor risk group a GMR was achieved by 38 of 110 pts (34.5%) after a median of 32.6 months (P=0.0089). To evaluate the prognostic significance of baseline mutations four groups were defined: (i) mutation-negative pts (n=57), (ii) pts harboring mutations reported to be sensitive to dasatinib in cell proliferation assays including those with no reported IC50 value (n=51), (iii) pts harboring mutations reported to confer a relevant loss of sensitivity to dasatinib (n=24; L248V, n=2; G250E, n=10; Q252H, n=2; E255K, n=3; E255V, n=2; F317L, n=4; F486S, n=1), and (iv) pts harboring T315I (n=3). Proportions of pts achieving GMR were 36.8%, 52.9%, 20.8% and 0.0% for groups i to iv, respectively. Median time to GMR resulted in one single significant difference, i.e. between group ii (10.3 months) and group iii (not defined, P=0.0247). Median pretreatment BCR-ABL levels of 41.1%, 40.1%, 40.3% and 42.7% for groups i to iv showed no significant difference indicating an independence of baseline mutation status and tumor load. *Conclusions.* The BCR-ABL tumor load in the situation of imatinib failure is highly predictive for the subsequent dasatinib response in CML-CP patients indicating the need for an early change in TKI regimen. Differences in BCR-ABL tumor load are not mediated by BCR-ABL mutations achieved under imatinib treatment.

0146

RESULTS FROM ENESTND: POPULATION PHARMACOKINETIC (PK) AND EXPOSURE-RESPONSE ANALYSIS OF NILOTINIB IN PATIENTS WITH NEWLY DIAGNOSED PH+ CHRONIC MYELOID LEUKEMIA IN CHRONIC PHASE (CML-CP)

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Background. Results from ENESTnd at 12 months demonstrated superior efficacy of nilotinib at doses of 300 mg BID and 400 mg BID over imatinib 400 mg QD. *Aims.* To perform a PK and exposure-response analysis of nilotinib in patients with newly diagnosed CML-CP. *Methods.* Full PK profiles of nilotinib were obtained at steady-state from a subgroup of 34 patients, and sparse (pre-dose/Cmin and 3-hr post-dose/Cmax) concentration data were obtained over 12 months from 542/563 (96%) treated patients. *Results.* Nilotinib concentrations were stable over 12 months in both arms (Cmin: 1158±568 ng/mL [median 1097 ng/mL] for 300 mg BID; 1340±621 ng/mL [median 1217 ng/mL] for 400 mg BID). The average nilotinib Cmin and Cmax were 15.7% and 14.8% higher in the 400 mg BID arm than the 300 mg BID arm. The estimated relative bioavailability ratio was 0.84 for nilotinib 400 mg BID to nilotinib 300 mg BID. Male patients had 10% lower bioavailability and systemic exposure than female patients. Although this difference was statistically significant, it was not considered clinically meaningful based on the observed interpatient variability in nilotinib PK. Age, body weight, ethnicity, and racial group did not significantly affect nilotinib PK. Adverse events on the study were typically transient and infrequently led to discontinuation of study drug. Total bilirubin elevation was higher in patients with higher nilotinib concentrations. For example, patients with nilotinib Cmin in quartiles Q1 (<829 ng/mL, n=113), Q2/3 (≥829 and ≤1569 ng/mL, n=229) and Q4 (>1569 ng/mL, n=113), had grade 3/4 bilirubin elevation of 0.9%, 4.8% and 7.1%, respectively. Logistic regression analysis confirmed a significant correlation between nilotinib daily AUC and total bilirubin elevation of all grades. There was no clinically relevant correlation between nilotinib exposure and elevation in AST or lipase. On average, an increase of 1000 ng/mL in nilotinib serum concentrations was associated with minimal increase in QTcF (4.2 to 6.9 msec). Although there was no apparent relationship between major molecular response at 12 months and average nilotinib Cmin, there was a weak positive correlation of uncertain significance (P = .097, Cochran-Mantel-Haenszel test for ordinal variables) between BCR-ABL transcript level reductions at 12 months and nilotinib Cmin (Table 1).

Table 1.

	Nilotinib C_{min}		
	< 829 ng/mL	≥ 829 and ≤ 1569 ng/mL	> 1569 ng/mL
	n = 113	n = 229	n = 113
	n (%)	n (%)	n (%)
Cumulative BCR-ABL (IS) ratio at 12 months			
≤ 0.0032%	1 (0.9)	10 (4.4)	8 (7.1)
≤ 0.01%	9 (8.0)	23 (10.0)	15 (13.3)
≤ 0.1%	45 (39.8)	101 (44.1)	52 (46.0)
≤ 1%	78 (69.0)	186 (81.2)	94 (83.2)
≤ 10%	93 (82.3)	206 (90.0)	100 (88.5)

Conclusions. There was a less than proportional dose-exposure relationship between nilotinib 300 mg BID and nilotinib 400 mg BID in the ENESTnd study. Exposure was stable over the 12 month treatment course at both doses. Demographics were not a clinically important

factor affecting nilotinib PK. The study is ongoing and additional PK data will be available.

0147

PHARMACOGENETICS OF IMATINIB IN NEWLY DIAGNOSED CHRONIC MYELOID LEUKEMIA PATIENTS SHOWS EVIDENCE OF ASSOCIATION BETWEEN IMATINIB TRANSPORTERS AND METABOLIZING ENZYMES GENOTYPE AND MOLECULAR RESPONSE

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Background. Imatinib mesylate (IM) is the first-choice treatment in chronic myeloid leukemia (CML), with excellent response rates. Major and possibly complete molecular responses are the current goals of therapy, but not all patients (pts) achieve these milestones - hence the need to find biological predictors of response in order to guide therapy optimization. *Aims.* We investigated if single nucleotide polymorphisms (SNPs) influencing IM delivery may account for differences in response. *Methods.* We have genotyped a panel of 20 SNPs affecting the activity of the cytochrome p450 isoforms (CYP3A4, CYP3A5) and of the transporters (ABCB1, ABCG2, hOCT1, OATP1A2, OCTN1) implicated in IM metabolism/transport in a subset of pts enrolled in the TOPS trial - a randomized phase III study of IM 400 mg/d vs IM 800 mg/d in newly diagnosed CML. A total of 189 pts were genotyped (156 Caucasian, 22 Asian and 11 non-Caucasian and non-Asian pts). Median follow-up time is 29 months. Additive models, with SNPs represented as the number of minor alleles, were used to assess association between individual SNPs and response outcomes. Summary measures based on SNPs from the same gene and those related functionally (uptake and efflux) were defined as the number of alleles, across the genes of interest, hypothesized to be associated with favorable response. The association of polymorphisms and SNP summary measures with complete cytogenetic response (CCgR) and major molecular response (MMR) at 12 months was assessed with exact Ps based on Cochran-Armitage trend tests. Survival analysis methods were used to examine the relationship between SNPs and cumulative incidence of major cytogenetic response (MCgR), CCgR, MMR, complete molecular response (CMR). Cumulative incidence curves were compared with log-rank tests of trend, assuming an additive genetic model. *Results.* Five SNPs (hOCT1: rs4646277, rs4646278, MDR1: rs1128501, Cyp3A4: rs28371759 and Cyp3A5: rs28365083) were homozygous for the major allele for all patients and were excluded from the analyses. Comparison of genotype frequencies according to the patient's demographic characteristics revealed differences between Asian and Caucasian subgroups. Among Caucasian, the following SNPs and/or haplotypes correlated with cumulative incidence of MMR: a) OCTN1 rs1050152 number (no.) of major alleles (A) (P=0.03); b) CYP3A4 rs2740574 no. of minor alleles (G) (P=0.04); c) hOCT1 gene total no. of major alleles (rs72552763, rs12208357, rs683369, rs2282143) (P=0.03); d) uptake (total no. of major alleles in hOCT1 rs72552763, rs12208357, rs683369, rs2282143, OCTN1 rs1050152, OATP1A2 rs11568563) (P=0.003). In addition, the following SNPs and/or haplotypes correlated with cumulative incidence of complete molecular response (CMR): a) MDR1 rs60023214 no. of major alleles (C) (P=0.005); b) uptake (total no. of major alleles in hOCT1 rs72552763, rs12208357, rs683369, rs2282143, OCTN1 rs1050152, OATP1A2 rs11568563) (P=0.009). *Conclusions.* Our results suggest that although ethnicity needs to be taken into account, pharmacogenetics of IM may be helpful in identifying pts who need treatment optimization. Confirmation in larger series is warranted to further test the predictive value of the SNPs identified in this study.

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0148

EUROPEAN SUB-REGISTRY OF CHRONIC MYELOID LEUKEMIA (CML) PATIENTS (PTS) IN FAILURE AFTER IMATINIB THERAPY (IFP): RATIONALE, STUDY DESIGN AND CURRENT STATUS. A STUDY FROM THE EUROPEAN LEUKEMIANET (ELN)

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Background. Since imatinib registration, most of the information on safety and efficacy of this therapy was collected through clinical trials. Thus, the context of imatinib failure is currently insufficiently documented. Therefore, members of the ELN established the IFP CML subregistry in order to collect failure pts. *Aims.* Failure is defined as 1) resistance, i.e. no haematological response (HR) or no cytogenetic response (CgR), unsatisfactory responses, loss of responses according to the ELN management recommendations (Baccarani, J Clin Oncol, 2009) and 2) toxicity leading to imatinib discontinuation. Objectives are to describe the categories of failure, demographic patterns, clinical and biological profiles of pts, treatments before failure, therapeutic decisions and outcome thereafter. *Methods.* To be included in the IFP sub-Registry, failure pts should be at least 18 years old with Ph^+ CML in chronic phase at the start of imatinib therapy. Eligibility was irrespective of treatments prior to imatinib and enrolment in clinical trials. Based on notification, data have been centralized since 2005 and 1000 cases are expected. *Results.* As of February 2010, 918 pts were registered by 23 IFP study groups from 16 countries (Austria, Czech Republic, Denmark, Finland, France, Germany, Israel, Italy, The Netherlands, Poland, Spain, Russia, Slovak Republic, Sweden, Switzerland and UK). Median age at diagnosis was 52 years (range: 12-88), 54% of pts being male. At the time of failure, 60% of pts have never been included in an imatinib clinical trial. Reasons for registration were: no or unsatisfactory response in 38% of the cases, including no complete HR (7%), no CgR at all (16%), and no major CgR (15%). Loss of responses occurred in a further 38% of the cases collected, including 14% of progression to accelerated phase or blast crisis. The remaining (24%) cases were recorded because of toxicity leading to imatinib discontinuation (20%) or toxicity combined with unsatisfactory responses (4%). With a median follow-up of 5 years since diagnosis (range: 1 month - 31 years), 769 cases were documented. The median duration of imatinib therapy was 25 months (range: 2 weeks - 103 months). Among these cases, 38% were previously treated with an interferon-based regimen. At the time of failure, 63% were treated with imatinib 400 mg alone or in combination with other drugs and 27% with various imatinib dosages. After failure, 47% of the pts have been switched to other tyrosine kinase inhibitors, 14% were transplanted, and 39% received several lines of treatment, high-dose chemotherapy and/or other compounds such as omacetaxine. At 8 years from imatinib start, the estimated rates of survival were 67% (95% CI: 60-71%) overall, 83% when toxicity leading

to imatinib discontinuation and 65% otherwise (P=0.015). *Conclusions.* This sub-registry is the first attempt to collect and provide detailed information about clinical features of patients who failed imatinib therapy, within and outside of clinical trials. The study is being conducted in accordance with the principles and guidelines of the European Community and is linked to the ELN. Completion of the data is ongoing. More detailed analyses will be presented.

0149

CML PATIENTS ACHIEVED CYTOGENETIC RESPONSE WITH LOW IMATINIB PLASMA LEVEL ARE CHARACTERIZED BY HIGH IMATINIB BONE MARROW FLUID LEVEL

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Background. Imatinib has shown the high effectiveness in chronic myeloid leukemia (CML) therapy. Recent papers have demonstrated that the achievement of complete cytogenetic response (CCyR) and major molecular response (MMR) to imatinib therapy may be relates with more than 1000 ng/mL Imatinib plasma level (IPL). However large inter-individual variability of IPL is peculiar for Imatinib-treated CML patients. Some patients achieve CCyR and MMR with significantly lower than 1000 ng/mL IPL. The Aim of this study is to compare the Imatinib bone marrow fluid level (IBMFL) with IPL in CML patients achieved CCyR and patients with failure or suboptimal response. *Methods.* IBMFL and IPL were detected in 170 CML patients with Imatinib treatment duration more than 12 months. The age of patients was 20-80. Male/female ratio was 73/97. 126 patients were in CP CML and 44 patients in AP CML. Imatinib doses were 400 mg QD (n=94), 600 mg QD (n=66) or 800 mg BD (n=10). Bone marrow and blood samples were collected simultaneously 1-72h after the last IM dose intake. Bone marrow samples were collected for cytogenetic analysis according to therapy protocol. 300 µL of each bone marrow sample were used for IBMFL test. All patients gave informed consent before sampling. Imatinib concentration were determined by a validated LC/MS/MS method. Exclusion criteria: imatinib dose 800 mg BD (10 pts), blood sampling less than 21 h (45 pts) and more than 27 h (13 pts), nonadherens (4 pts), the dilution of BM samples by blood (bone marrow cell count $<20\times10^9$/L , M/E ratio <2)(28 pts). Totally 68 patients were included in analysis. *Results.* Cytogenetic analysis have revealed that 42 patients have achieved CCyR and 26 have achieved suboptimal response or failured to achieve cytogenetic response. The median of IPL in patients with CCyR was 1135 [536-2519] ng/mL, IBMFL - 1818 [708-5827] ng/mL , IBMFL/IPL ratio was 1,8 [0,7-3,4]. The median of IPL in patients with failure or suboptimal response was 1069 [197-1963] ng/mL, IBMFL - 1647 [250-3959] ng/mL, IBMFL/IPL ratio was 1,3 [0,8-3,6]. Among 42 patients achieved CCyR IPL more than 1000 ng/mL were revealed in 26 (61,9%) patients with the median of IPL - 1547 [1021-2519] ng/mL, IBMFL - 2701 [1175-5827] ng/mL, IBMFL/IPL ratio - 1,7[0,9-3,4] . Less than 1000 ng/mL were founded in 16 (38,1%) patients achieved CCyR with the median of IPL - 731 [536-998] ng/mL, IBMFL - 1342 [708-2735] ng/mL, IBMFL/IPL ratio - 1,8 [0,7-2,8]. Among 16 patients achieved CCyR with low IPL (<1000 ng/mL) IBMFL is more than 1000 ng/mL and significantly exceed IPL in 14 (87,5%) patients with IBMFL/IPL ratio 1,9 [1,4-2,8]. *Conclusions.* Imatinib level in bone marrow fluid is higher than in blood plasma in most cases. The achievement of CCyR in cases with low level of imatinib concentration in plasma is associated with higher level of imatinib in bone marrow.

0150

HOCT1 M420 DELETED TRANSCRIPT LEVELS PREDICT POOR CLINICAL OUTCOME FOR IMATINIB TREATED CML PATIENTS

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Background. Human organic cation transporter 1 (hOCT1) transports imatinib into haematological cells. However, whilst we and others have shown that hOCT1 transcript levels are predictive of outcome in imatinib treated CML, this is not universally agreed. These variable results may be because differing PCR primer locations have variable detection of functionally relevant single nucleotide polymorphisms (SNPs) in hOCT1. Here we focus on a 3-nucleotide deletion at M420, the commonest hOCT1 SNP in the general population (allele frequency 14-19%) and which is known to modify hOCT1 mediated metformin transport. *Methods.* In our original primer set (Wang et al, Clin. Pharm. Therap. 2008), the forward primer covers the M420del SNP site. New primers were designed with similar PCR efficiency shifted away from M420del SNP. Both pairs of primers were then used for hOCT1 transcript quantification on 13 "test"and 53 "validation"CML samples, using optimised real time PCR conditions on a LightCycler. *Results.* In 5 cases without M420del, equal amounts of hOCT1 transcripts were detected by the two sets of primers. In 6 cases heterozygous for M420del, the apparent hOCT1 transcript levels measured by the original primers were 50% of those measured by the new primers. In 2 cases homozygous for M420del, hOCT1 transcripts were undetectable by the original primers, though were detectable by the new primers at levels comparable to M420 wild type cases. The original primers therefore detect purely M420 wild type transcripts, while the new primers detect both M420 wild type and M420del transcripts, which was confirmed by sequencing the PCR product. To assess whether M420del SNP predicts clinical outcome, hOCT1 transcript levels were measured using both sets of primers in 53 "validation"newly diagnosed chronic phase patients who then received first line imatinib. M420del SNP were determined by PCR pyrosequencing and cloning sequencing. In cases heterozygous for M420del, apparent transcript levels were significantly lower with the original than with the new primer sets (P=0.03), whereas little difference was seen in cases without M420del. Using the original primer set, patients achieving complete cytogenetic response (CCR) after 12 months of imatinib treatment had higher apparent transcript levels than patients with no cytogenetic response (NCR) (P=0.01). In contrast, using the new primer set, no significant difference in transcript levels was seen between CCR vs. NCR patients. Survival analysis showed that patients with high transcript levels as defined by the original primers had superior event free survival (EFS) and progression free survival (PFS) than cases with low apparent expression (P=0.03 and 0.01, respectively. Log-rank analysis); however, with the new primers, EFS and PFS were not significantly different between patients with high vs. low transcript levels. *Conclusions.* These data illustrate that a) M420 wild type transcript levels (as measured by our original primers) correlate with the outcome of treatment while actual hOCT1 transcript levels (as assessed here by our new primers) may not, and b) M420del may be a clinically important SNP that predicts outcome of imatinib treatment. Further investigation of the role of hOCT1 M420del SNP is required.

0151

DETECTION OF NEW MUTATIONS IN NILOTINIB-TREATED PATIENTS WITH IMATINIB-RESISTANT CHRONIC MYELOID LEUKEMIA IN CHRONIC PHASE

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Background. Nilotinib is a potent and the most selective BCR-ABL kinase inhibitor, approved for the treatment of chronic phase (CML-CP) and accelerated phase (CML-AP) Philadelphia chromosome positive (Ph^+) CML in adult patients resistant to or intolerant of prior therapy, including imatinib. BCR-ABL transcript levels and the detection of new mutations have been reported to be a predictor of response outcomes in patients with CML, including those with imatinib resistance (Branford et al, Blood. 2008;112(11):128). *Aims.* Here we examined the association between newly detectable BCR-ABL mutations, presence or absence of baseline mutations, resistance to or intolerance of imatinib, and initial molecular response to nilotinib in patients with Ph^+ CML-CP treated in the phase 2 nilotinib registration study 2101. *Methods.* Patients with Ph^+ CML-CP (N = 321) with imatinib resistance (78%) or intolerance (22%) were included in this post-hoc analysis. All patients had at least 24 months of follow-up. Landmark analyses were performed based on BCR-ABL transcript levels at 3 months (≤1%, > 1-≤10%, and > 10% BCR-ABL (International Scale, IS). *Results.* Newly detectable mutations were observed in 56 of 290 (19%) patients with available baseline mutation data, including 51 of 200 (26%) imatinib-resistant and 5 of 90 (6%) imatinib-intolerant patients. Patients with baseline mutations and resistance to prior imatinib had a higher probability of newly detectable mutations compared with patients without baseline mutations (P < .0001) and imatinib-intolerant patients (P=.0002). In patients with baseline mutations or those resistant to imatinib therapy, BCR-ABL levels at 3 months did not predict for newly detectable mutations, including mutations less sensitive to nilotinib (Y253H, E255K/V, F359C/V, T315I) (Table). Molecular response to nilotinib treatment at 3 months also did not predict for newly detectable mutations in patients without baseline mutations and those intolerant of imatinib. *Conclusions.* In this analysis, detection of new mutations was more likely in patients with baseline mutations and imatinib resistance. Molecular response to nilotinib at 3 months did not correlate with detection of new mutations within 24 months of initiating nilotinib therapy. Other factors predictive of newly detectable mutations should be explored.

Table 1. Occurrence of newly detectable mutations by 24 months according to BCR-ABL trascription levels at 3 months on nilotinib.

	BCR-ABL levels at 3 Months (IS, %)			
	≤ 1	> 1-≤ 10	> 10	P-value
Occurrence of newly detectable mutations by 24 mo (%)				
Pts with baseline mutations, n = 79	21	17	45	.258[a], .983[b]
Resistant pts, n = 158	21	14	28	.146[a], .532[b]
Occurrence of less sensitive mutations by 24 mo (%)				
Pts with baseline mutations, n = 79	21	17	21	.983[a], .919[b]
Resistant pts, n = 158	11	10	16	.556[a], .858[b]

[a]Comparison of > 1-≤10 vs > 10
[b]Comparison of ≤ 1 vs > 1-≤ 10

Clinical thrombosis

0152

RECURRENT VENOUS THROMBOEMBOLISM AND POST-THROMBOTIC SYNDROME AFTER CATHETER-DIRECTED THROMBOLYSIS IN DEEP VENOUS THROMBOSIS

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Background. In the present study we investigated the risk of recurrence in patients who were given additional catheter directed thrombolytic therapy (CDT) to conventional treatment with heparin for severe proximal deep vein thrombosis (DVT). We have shown that such treatment significantly increases recanalization and patency. Based on recent studies suggesting higher risk of recurrence in patients with residual vein thrombosis, we hypothesized that CDT would be associated with lower risk of recurrence. *Aims.* To determine the rate of VT recurrence and frequency of post-thrombotic syndrome (PTS) following treatment with CDT. *Methods*. We identified patients treated with CDT in 4 hospitals in the Norwegian South-Eastern Health Region during 1999-2006. Sixty-eight consented to participate in this study. Patients met for investigation during May 2009 -Jan 2010. PTS was assessed by the Villalta score. *Results.* Median age (inter-quartile range =IQR) of 68 patients was 41 (33-56) years; 51 females (75%). Median duration of follow-up was 6.3 years (IQR 4.8-8.1 years). Recurrent VT was encountered in 5 of 21 (24%: 95% CI 9-47.5%) on AC, and in 10 of 47 (21%: 95% CI 11-36%) after discontinuing AC. The recurrences were DVT in 13 (86%), Pulmonary embolism in 1 (7%) and retinal vein thrombosis in 1 (7%). The 10-years probability of VT recurrence in all patients after discontinuation of AC is estimated to 32%. DVT was classified as provoked in 34 (71%) and unprovoked in 14 (29%). Significantly higher recurrence rate was seen in patients with unprovoked compared to provoked DVT (log rank test P=0.008) (Figure). Elastic compression stockings (ECS) were used in 60 patients (88%). According to Villalta score 43 patients (64%: 95% CI 51-75%) had no PTS; 15 (22%: 13-34) had mild, 6 (9%: 4-19) had moderate and 3 (4%: 1-13) had severe PTS. *Summary/Conclusions.* This study provides the longest follow-up data reported on rates of recurrence and PTS after CDT. The 10-years probability of VT recurrence after discontinuation of AC is estimated to 32%, which is in line with that found after conventional anticoagulation (Prandoni et al: Haematologica 2007;92). The incidence of PTS in this study was comparable to that reported following conventional treatment (Schulman. J Thromb Haemost 2006;4). In conclusion, there is no indication that CDT provides any benefit over conventional treatment in term of reduction in VT recurrence or PTS

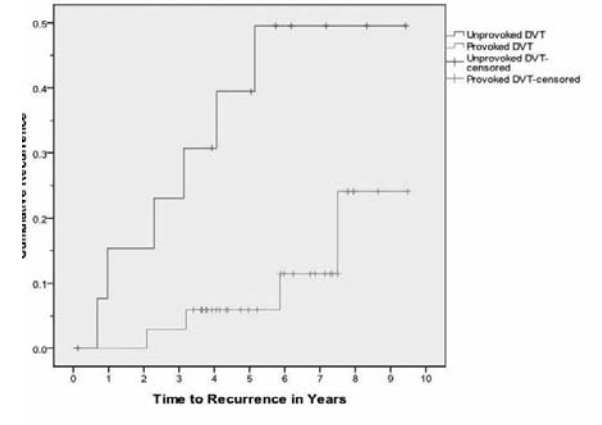

Figure. Recurrence in provoked and unprovoked DVT.

0153

BODY HEIGHT AND SEX-RELATED DIFFERENCES IN INCIDENCE OF VENOUS THROMBOEMBOLISM: A DANISH FOLLOW-UP STUDY

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Background. Pulmonary embolism (PE) and deep venous thrombosis (DVT) share many risk factors, and most studies regard these diseases as one entity namely venous thromboembolism (VTE). However, it is well known that people who have had a PE event recur as PE more frequently than patients who have had a primary DVT event indicating that some people are more predisposed for PE than others, i.e. some people are for some reasons more likely to embolise from a DVT into PE. It is unknown whether sex influences the presentation of VTE as DVT or PE and the impact of body height on the presentation of VTE has not been evaluated. *Aim.* to compare the incidence rate of VTE, DVT and PE between men and women and to clarify to which extent any sex-related differences are mediated by body height and other known risk factors for VTE. *Methods.* We computed sex-specific incidence rate of VTE, DVT and PE and estimated the crude and adjusted incidence rate ratios of VTE, DVT and PE using Cox regression for men versus women participating in the prospective study Diet, Cancer and Health. We controlled for body mass index, body height, leisure-time physical activity and smoking dose. *Results.* In total, 56,014 people were followed for a median of 10.2 years during which 641 incident VTE events were verified. Table 1 shows the incidence rates of VTE, DVT and PE, including idiopathic and provoked events, in men and women, and the crude and adjusted incidence rate ratios for men versus women. The crude IRR was 2.00 [95% CI: 1.61-2.49] for DVT and 1.14 [95% CI: 0.90-1.44] for PE for men versus women, showing that the crude incidence rate of DVT was double as high in men than women, but no difference in the crude incidence rate of PE were found between the two sexes. In contrast, the adjusted incidence rate ratios were 1.06 [95% CI: 0.75-1.50] for DVT and 0.60 [95% CI: 0.41-0.87] for PE for men versus women. The main confounder in the analyses was body height whereas adjustment for BMI, smoking status, and physical activity only resulted in minor changes of the estimates. This shows that men and women at the same body height, BMI, smoking status, and level of physical activity had the same risk of DVT, whereas the risk of PE were significant higher in women. The same tendency was found for idiopathic and provoked VTE. *Summary/Conclusions.* In conclusion, in this middle-aged population, men experienced a higher incidence of VTE due to a higher incidence of DVT. The higher incidence among men appeared to be mediated mainly by body height. Controlled for body height, the incidence of PE was notably lower among men compared with women.

Table 1.
Incidence rates (IR) and incidence rate ratios (IRR, i.e. IR_{men} / IR_{women}) of venous thromboembolism (VTE), deep venous thrombosis (DVT), and pulmonary embolism (PE) per 1000 person years at risk. Adjusted for BMI, body height, physical activity, smoking status. [95 % confidence intervals].

	IR, total [95% CI]	IR, men [95% CI]	IR, women [95% CI]	IRR, crude [95% CI]	IRR, adjusted [95% CI]
VTE, all (n=641)	1.15 [1.07-1.25]	1.40 [1.27-1.56]	0.92 [0.82-1.04]	1.55 [1.32-1.82]	0.82 [0.63-1.05]
DVT, all (n=358)	0.65 [0.58-0.72]	0.87 [0.76-0.99]	0.44 [0.37-0.53]	2.00 [1.61-2.49]	1.06 [0.75-1.50]
PE, all (n=283)	0.51 [0.45-0.57]	0.54 [0.45-0.64]	0.48 [0.41-0.57]	1.14 [0.90-1.44]	0.60 [0.41-0.87]
VTE, idiopathic (n=505)	0.55 [0.49-0.61]	0.72 [0.62-0.83]	0.39 [0.32-0.47]	1.88 [1.48-2.37]	1.01 [0.70-1.45]
DVT, idiopathic (n=169)	0.30 [0.26-0.35]	0.46 [0.38-0.55]	0.16 [0.12-0.22]	2.84 [2.02-3.99]	1.43 [0.85-2.41]
PE, idiopathic (n=135)	0.24 [0.21-0.29]	0.26 [0.21-0.33]	0.23 [0.18-0.29]	1.18 [0.84-1.67]	0.70 [0.41-1.18]
VTE, provoked (n=311)	0.55 [0.49-0.61]	0.62 [0.53-0.72]	0.48 [0.40-0.56]	1.33 [1.06-1.67]	0.69 [0.48-1.00]
DVT, provoked (n=186)	0.34 [0.29-0.39]	0.41 [0.33-0.49]	0.27 [0.22-0.34]	1.51 [1.12-2.02]	0.83 [0.52-1.32]
PE, provoked (n=116)	0.21 [0.17-0.25]	0.21 [0.16-0.28]	0.20 [0.16-0.26]	1.09 [0.75-1.58]	0.52 [0.29-0.94]

0154

IN KINDREDS WITH INHERITED THROMBOPHILIA THE VENOUS THROMBOTIC RISK OF THE CARRIERS IS DEPENDENT ON THE CLINICAL PHENOTYPE OF THE PROBAND

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Background. Laboratory screening for inherited thrombophilia is recommended for patients with previous venous thromboembolism (VTE) or obstetric complications (OC). Universal screening (US) before oral contraceptive intake or pregnancy is discouraged, being not cost-effective. Nevertheless, a growing number of individuals are labeled as carriers of inherited thrombophilia because of inappropriate laboratory testing. Whether the identification of all the asymptomatic affected relatives should be pursued in order to prevent VTE is debated. *Aims.* To assess in kindreds with inherited thrombophilia the thrombotic risk of the carriers according to the type of the alteration and to the clinical phenotype of the proband. *Patients and Methods.* We carried out a retrospective family cohort study on 1,722 relatives (M/F 769/953) of 563 probands diagnosed as carrying thrombophilia because of a history of VTE (1,090 relatives from 340 kindreds), OC (257 relatives from 86 kindreds), premature arterial thrombosis (AT) (113 relatives from 31 kindreds), or US (262 relatives from 106 kindreds). In 517 kindreds the proband carried factor V Leiden (FVL) and/or prothrombin G20210A (PTGA), whereas in the remaining 46 the proband had a deficiency of antithrombin (AT), protein C (PC) or S (PS), alone or combined with FVL or PTGA; all the probands had been referred for counselling and familial screening. The relative risk for deep venous thrombosis (DVT) was estimated as a hazard ratio (HR) comparing the thrombosis-free survival curves of the carriers in respect to the non-carriers. *Results.* Inherited thrombophilia was detected in 968 relatives (56.2%); the total observation-years were 37,727 for carriers and 29,548 for non-carriers. DVT occurred in 46 carriers (incidence 1.2 per 1,000 individual-years) and in 11 non-carriers (incidence 0.37 per 1,000 individual-years). Overall, the risk for DVT was significantly increased in the carriers only in the kindreds of the probands diagnosed because of previous VTE (HR 2.40, 95%CI 1.14-4.05). No increase in risk was detectable among the carriers who were relatives of the probands diagnosed because of OC, AT, or US, whatever the type of alteration present in the kindred. In the kindreds of the probands with FVL and/or PTGA and previous VTE, the risk for DVT in the carriers was marginally increased (HR 1.97, 95%CI 0.89-3.92). In the kindreds of the probands with deficiency of natural anticoagulants (AT, PC, or PS) and previous VTE, the HR for DVT in respect to the non-carriers was 4.66 (95%CI 0.80-10.21) in the overall carriers, and 15.36 (95%CI 3.06-60.91) in the relatives with AT deficiency. *Conclusions.* Familial screening for inherited thrombophilia is only justified for probands with history of VTE. In this setting, the risk for DVT is about doubled in the carriers of FVL and/or PTGA, and is exceedingly high in the carriers of AT deficiency.

0155

HEMOSTATIC FACTORS AND MORTALITY BY ACUTE LUNG INJURY IN SEVERE SEPSIS SYNDROME

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Background. Sepsis often presents disseminated intravascular coagulation and acute lung injury (ALI) and acute respiratory distress syndrome (ARDS), related to severity of the disease. The aim of this study is to assess the dynamics and magnitude of the coagulation and fibrinolysis plasma markers in the severe sepsis syndrome and the relation with mortality associated to ALI / ARDS. *Methods.* 2619 patients in intensive care units of 13 hospitals were screened at admission and daily for severe sepsis, and presence of ALI / ARDS. 311 septic patients were included during a 6 months period, according to definition of ACCP Consensus Conference 1992 and Resp Critical Care Consensus Conference 1994. The state of the coagulation system was assessed by measurements of values of thrombin-antithrombin complex (TAT), and Protein C (PC). The fibrinolytic system was assessed by the measurement of plasmin-antiplasmin complex (PAP) and plasminogen-activator inhibitor type 1 (PAI-1). Enzyme linked immuno-assay methods in plasma were used. The samples were collected at diagnosis, and at days 3

and 7 from the first episode of severe sepsis in a random sample of 145 patients. Non-parametric U Mann-Whitney test stablished differences between patients with ALI / ARDS and the other septic patients, and between survivors with non survivors ALI / ARDS patients. *Results.* There were 92 (63,5%) patients with diagnostic of ALI / ARDS during the first seven days after admission, and 63 (68,5%) died. Plasma concentrations of fibrinolytic inhibitor PAI-1 were higher at day 1 in patients with the presence of lung injury (91.6 vs 50.7, P=0.04). Non-survivors ALI / ARDS patients had higher levels of PAI-1 at day 1 (91.6 vs 50.2, P=0.02) and day 3 (63.2 vs 45, P=0.01). Patients with ALI / ARDS during all the first week had higher levels of PAI-1 at day 1. When lung injury appeared at day 3 or 7, previous higher levels of PAI-1 at day 1 (154 vs 46.5, P=0.03) or day 3 (126 vs 30, P=0.01) were associated to mortality. No other hemostatic markers analized had significative relation with the presence of ALI / ARDS. *Conclusions.* High plasma concentration of fibrinolytic inhibitor PAI-1 is correlated with the presence of lung injury / acute respiratory distress syndrome in septic patients. PAI-1 could be considered an mortality predictor in patients with severe sepsis and ALI / ARDS.

0156

SPLENECTOMY AND THROMBOSIS: THE CASE OF THALASSEMIA INTERMEDIA

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Background. The pathophysiological mechanisms that lead to the hypercoagulable state in splenectomized patients with thalassemia intermedia (TI) have been identified. However, clinical characteristics of patients who eventually develop overt thromboembolic events (TEE) are poorly studied. *Aims.* To identify factors that characterize splenectomized TI patients who develop TEE as compared to splenectomized and non-splenectomized patients who do not develop TEE. *Methods.* Data was retrieved from the Thalassemia Intermedia Registry, a database of 584 TI patients currently registered at six comprehensive care centers in Lebanon, Italy, Iran, Egypt, United Arab Emirates, and Oman. Institutional review boards at each center approved the study protocol. Three Groups of patients were identified: Group I, splenectomized patients with a documented TEE (n=73); Group II, age- and sex-matched splenectomized patients without TEE (n = 73); and Group III, age- and sex-matched non-splenectomized patients without TEE (n=73). Collected data included demographics, laboratory parameters, disease-complications, and received treatments that may influence TEE development and reflected the period prior to TEE occurrence in Group I. *Results.* The mean age was 33.1±11.7 years (range, 15-60 years) for Group I (at TEE), 33.3±11.9 (range, 9-75 years) for Group II, and 33.4±13.1 (range, 6-76 years) for Group III; (P=0.991). The male to female ratio was 33:40, 35:38, and 34:39 for Groups I, II, and III; respectively (P=0.946). There was no statistically significant differences in mean hemoglobin (Group I: 9.0±1.3 g/dL, Group II: 8.8±1.2 g/dL, Group III: 8.7±1.3 g/dL; P=0.174) or fetal hemoglobin (Group I: 45.9±28.0%, Group II: 54.4±32.8 g/dL, Group III: 44.2±27.2 g/dL P = 0.429) levels between Groups. However, nucleated red blood cell (RBC) counts were highest among Group I (mean, 410.8±299.2×10^6/L), followed by Group II (mean, 301.0±130.7×10^6/L), then Group III (mean, 241.0±130.5 ×10^6/L); P = 0.015. Similarly platelet counts were highest among Group I (mean, 712.6±192.5×10^9/L), followed by Group II (mean, 506.3 ±142.1×10^9/L), then Group III (mean, 319.8±125.5×10^9/L); P<0.001. There was no statistically significant difference in the proportion of patients with diabetes mellitus, heart failure, abnormal liver function, or prothrombotic mutations. However, a higher proportion of patients had pulmonary hypertension in Groups I (31.5%) and II (28.8%) as compared to Group III (2.7%); P<0.001. The highest proportion of patients receiving transfusion therapy was in Group III (74%), followed by Group II (65.8%), then Group I (43.8%). There was no statistically significant difference in the proportion of patients receiving aspirin, anticoagulants or hydroxyurea between the three Groups. Multivariate logistic regression analysis revealed that patients in Group I are more likely to be non-transfused, have pulmonary hypertension, have nucleated RBC counts > 250×10^6/L, and platelet counts > 500×10^9/L. *Summary/Conclusions.* Transfusion naivety, pulmonary hypertension, and high platelet and nucleated RBC counts characterize splenectomized TI patients who develop TEE.

0157

VENOUS THROMBOEMBOLISM DURING AUTOLOGOUS STEM CELL TRANSPLANTATION FOR HAEMATOLOGICAL MALIGNANCIES

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Background. There is an established risk of venous thromboembolism (VTE) with solid tissue malignancies. Recent evidence indicates an increased risk also with haematological malignancies, including during myelosuppressive therapy for acute leukaemia. Current guidelines suggest pharmacological prophylaxis in hospitalised cancer patients, but the incidence of VTE during autologous stem cell transplantation is unknown, and there are potential risks of pharmacological VTE prophylaxis during myeloablative chemotherapy. *Aim.* To establish the risk of VTE during autologous stem cell transplant. *Method.* A retrospective review of all patients undergoing autologous stem cell transplant for haematological malignancies at our institution was undertaken to determine the incidence of radiologically confirmed symptomatic VTE and the presence of concurrent risk factors. *Results.* One hundred eighty four patients had follow up for at least one month following discharge and were included in the analysis. There were two deaths within the follow-up period (1 graft failure, 1 progressive disease). Non-Hodgkin lymphoma (NHL) and myeloma were the most common indications for transplant, with BEAM and melphalan, respectively the most common conditioning regimens. Twelve patients had a history of prior VTE, of whom three had pharmacological prophylaxis, which was withheld during severe thrombocytopenia in two cases.A total of four (2.2%) patients had confirmed VTE. None had had a prior event. Three events were related to central venous catheters. Only one patient (0.5%) had a VTE not associated with a venous catheter, developing an above knee deep vein thrombosis after discharge. Two of 9 patients with peripherally inserted central catheters (PICC) developed catheter associated thrombosis, which was the only significant risk factor. No patients in this cohort had veno-occlusive disease of the liver. *Conclusions.* Autologous stem cell transplant with BEAM or melphalan has a low risk of venous thromboembolism and pharmacological prophylaxis is not recommended. The use of PICC lines carried a higher risk of thrombosis.

0158

CLINICAL CHARACTERISTICS AND OUTCOME OF PATIENTS WITH FACTOR V LEIDEN OR PROTHROMBIN G20210A AND A FIRST EPISODE OF VENOUS THROMBOEMBOLISM. FINDINGS FROM THE RIETE REGISTRY

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Background. Asymptomatic individuals with factor V Leiden or prothrombin G20210A are at a higher risk to develop venous thromboembolism (VTE), but little is known about the clinical characteristics of the VTE in these patients. *Patients and Methods.* RIETE is an ongoing registry of consecutive patients with acute VTE. Our aim was to compare the clinical characteristics and outcome of those with a first episode of VTE and factor V Leiden, prothrombin G20210A, or no thrombophilia. *Results.* As of May 2009, 22428 patients had been enrolled with a first episode of VTE. Of these, 345 had factor V Leiden, 261 prothrombin G20210A, 2399 tested negative. Sixty-two percent of VTE episodes in women were associated with an acquired risk factor, 40% in men. Among women with factor V Leiden or prothrombin G20210A, contraceptive use and pregnancy accounted for 63% and 67% of such risk factors. Patients with factor V Leiden presented with pulmonary embolism less often (31% vs. 51% or 45%, respectively), and only had Sat O2 levels <90% less frequently (4.5% vs. 17% and 20%, respectively). There were no differences between subgroups in the incidence of recurrent VTE, either during or after discontinuation of anticoagulant therapy. *Conclusions.* Most episodes of VTE in women (not men) with factor V Leiden or prothrombin G20210A were associated with an acquired risk factor (mostly preg-

nancy or contraceptive use). Only a minority of patients with factor V Leiden presenting with acute PE had hypoxaemia.

0159

ROLE OF PROCOAGULANT MICROPARTICLES IN ARTERIAL ATHEROTROMBOSIS IN PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS

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Background. Microparticles (MPs) are a key component in the haemostatic response. *Aim.* To analyze the procoagulant activity of MPs and its correlation with arterial thrombosis and preclinical atherosclerosis in patients with systemic lupus erythematosus (SLE). *Methods.* We included 100 patients with SLE (27 with associated APS, 36 with antiphospholipid antibodies -aPL- but without APS and 37 without aPL). Early atherosclerosis was evaluated by ultrasonographic study of carotids measuring intima-media wall thickness and presence of arteriosclerotic plaque. Procoagulant MPs were assessed by a functional assay in which MPs were captured through annexin V and then thrombin was formed by the addition of activated factor X, activated factor V and prothrombin (Hyphen BioMed, Neuville, France). *Results.* A total of 16 episodes of arterial thrombosis in 8 patients with APS, 7 without aPL and 1 patient with aPL have been registered. SLE patients with associated APS had greater prevalence of plaque than patients without aPL or with aPL but without APS (51.9% vs 24.3% vs 22.2%). No differences were seen in procoagulant MPs according the gender or the age of the patients. Overall, there was a significant relationship between procoagulant MPs and the presence of carotid plaque (16.7±8.6 nM vs 12.7 ±7.3 nM; P=0.02) and the number of carotid plaques (14.5±4.0 nM in patients with one plaque and 17.3±10.3 nM in patients with 2 or more plaques; P=0.02). In addition, a relationship between arterial thrombotic events and the procoagulant activity (18.7±9.5 nM with events vs 13.0±7.2 nM without events, P=0.007). *Conclusions.* Procoagulant MPs may be implicated in arterial thrombosis and atherosclerosis in SLE patients.

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0160

THROMBOSIS IN RELATION TO VON WILLEBRAND FACTOR, VWF PROPEPTIDE AND ADAMTS13 PROTEASE LEVELS IN PHILADELPHIA CHROMOSOME NEGATIVE MYELOPROLIFERATIVE NEOPLASMS

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Background. Thrombohemorrhagic events are the main cause of mortality and morbidity in Philadelphia chromosome negative myeloproliferative disorders (MPNs). Hyper viscosity and activated endothelium due to interaction of the elevated cellular components together with the comorbitities arising from patients older age compose a thrombophilic environment. The JAK2V617F mutation further contributes to this hypercoagulable state as it is related to high white cell count and platelets adhesion and activation. Von Willebrand factor (vWF) which is cleaved by ADAMTS13 protease plays central role in hemostasis while ppvWF is a good indicator of vWF synthesis. *Aim.* We measured vWF and ppvWF levels and ratio (ppvWF/vWF) as well as ADAMTS13 activity in MPNs steady state patients (no event for at least 6 months prior to evaluation).We evaluated their fluctuation in terms of thrombosis manifestation and identified risk factors for thrombotic events. *Methods.* A total of 37 patients, 18 males/19 females, with median age 53(20-81) and median follow up period 76 m (3-140) were evaluated. Eleven were diagnosed with polycythemia vera, 22 with essential thrombocytosis and 4 with primary myelofibrosis. Control group consisted of 92 healthy blood donors after written agreement. All clinical and laboratory data were concurrently recorded. ADAMTS13 activity was measured by fluorescent technique (FRETS), vWF and ppvWF antigen levels by ELISA (U/dl).We used Real Time PCR to assess JAK2V617F mutation status. All patients were screened for VLeiden and Pr20210 mutations by PCR- based techniques. *Results.*We found lower ADAMTS activity in patients plasma compared to control group (99.4% and 125% respectively, P<0.001). Inverse correlation was noticed between ADAMTS and vWFAg (P<0.05) in patients while the latest was strongly correlated with ppvWF levels (P<0.001).).Thrombotic events occurred in 11/37 patients (7 CNS/2 DVT/1 BuddChiari). JAK2V617F was carried by 22/37 and only 1 was heterozygous for VLeiden. Acquired Von Willebrand Syndrome was not noticed. Higher levels of vWF were measured in patients with thrombotic history compared both to healthy group (P<0.003) and patients without thrombosis (mean vWFAg 133.82 and 77 U/dl respectively).In multivariate analysis, JAK2V617F mutation (P<0.036), vWFAg steady state levels (P<0.030) and ratio (ppvWF/vWF) (0.044) were independent factors related to thrombosis. *Summary*. In our study group vWFAg steady state levels strongly correlated with thrombosis. The (ppvWF/vWF) ratio differentiated in terms of thrombotic history, suggesting that in MPN patients with thrombosis both synthesis and clearance of mature vWF were equally affected while in patients not suffering of thrombosis increased vWF clearance may balance hypercoagulable diathesis. We found lower ADAMTS13 activity, yet not abnormal in MPNs patients. In agreement with current literature JAK2V617F was found independent risk factor related to thombosis. There is need for prospective follow up of the measurements done in order to establish their prognostic value in steady-state Philadelphia Chromosome Negative Myeloproliferative Neoplasms.

0161

LEVELS OF CIRCULATING MICROPARTICLES IN PATIENTS WITH GYNAECOLOGICAL MALIGNANCY - A CASE-CONTROL STUDY

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Background. Microparticles (MPs) are important biological effectors, considered to be responsible for several patho-physiological processes. They carry surface antigens and proteins that allow inter-cellular signalling, and they are thought to have an important role in prothrombotic conditions, being able to support and also initiate thrombosis through their surface expression of negatively charged phospholipids +/- tissue factor respectively. It is well known that cancer patients are at an increased risk of venous thromboembolism (VTE), approximately 4 to 5 times more than the general population, and patients with cancer who suffer a VTE have a worse prognosis from a malignancy point of view. Being able to accurately predict the risk of VTE would allow patients at a high risk of VTE to be treated with primary thromboprophylaxis, potentially improving their malignancy-related prognosis. The level of circulating MPs, especially platelet derived MPs, has been reported to be elevated in several prothrombotic disorders, including malignancy. *Aims.* To compare the level of circulating MPs in patients with gynaecological malignancy with the level of MPs in a control group of patients with benign gynaecological conditions. We also wanted to establish if the level of MPs was predictive of the incidence of VTE. *Methods.* We recruited 64 patients with gynaecological malignancy and 45 control patients with benign gynaecological conditions. Written informed consent was obtained from all patients prior to taking blood samples. The MPs were isolated using flow cytometry. The MPs and their cell of origin were positively identified by gating on size (<1µm) and positivity for cell specific antigens (CD41 - platelet MPs (PMPs), CD144 - endothelial MPs (EMPs), CD45 - leucocyte MPs (LMPs). We also measured the total number of tissue factor (TF) (CD142) positive MPs. All the MP analysis was done on fresh plasma samples, within 2 hours of collection. *Results.* The median level of circulating PMPs, EMPs, LMPs and TF+ve MPs was similar in the patient and control group with no statistically significant difference: PMPs 1407(161-24014)/µL vs 1164 (58-16,111)/µL [P=0.22], EMPs 636(104-5177)/µL vs 506(109-8362)/µL [P=0.51], LMPs 1513(137-12,335)/µL vs 1308(210-11,702)/µL [P=0.81], TF+ve MPs 814(88-5506)/µL vs 909(121-3518)/µL [P=0.68]. Only 5 (7.8%) of the patients with gynaecological malignancy were diagnosed with a VTE event (none of the control group suffered a VTE event); again there was no statistically significant difference between the malignant patients with VTE and the malignant patients without VTE (P=0.13 for PMPs) however the number of patients diagnosed with a VTE is too small to draw firm conclusions. *Summary*. In conclusion the circulating MP level was similar in patients with gynaecological malignancy and in women with benign gynaecological conditions. Larger numbers of patients with both gynaecological malignancy and VTE are needed to establish any correlation between the level of MPs and the risk of VTE.

0162

ORAL ANTICOAGULANT THERAPY (OAT) IN PATIENTS WITH FONTAN SURGERY

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Thrombotic events have been reported as a major cause of morbidity after the Fontan procedure. There is no consensus about the type and duration of postoperative anticoagulation prophylaxis, due to the high risk of bleeding complications, the difficulties in monitoring and the questionable therapeutic compliance in children. In spite of the lack of long term prospective studies in this situation, the ACCP has recommended in their guidelines OAT following Fontan or Glenn operation for at least 6 months. This has also been our practice in our institution during the period of study. *Aim of the study.* To analyze the efficacy and complications of OAT in our pediatric patients after undergoing the Fontan operation. *Methods.* Retrospective chart review of all the children treated with OAT in our institution between 1995 and 2009. All patients were treated initially with acenocumarol 0,2 mg/kg, except the Fontan patients, who received 0,1 mg/kg. Target INR was 2-3 for all patients, except for prosthetic valve recipients, in which target INR was 2,5-3,5.The Mann-Whitney test was used to compare the rate of complications, and the percentage of visits out of target INR between the Fontan patients and the rest of the cohort. *Results.* There were 68 children (29 female/ 39 males) aged between 1 month and 17 years, who received OAT with a range of follow up between 4 months and 14 years: 27 after Fontan operation (Group A), an 39 for other reasons (Group B: n=6 prosthetic heart valves, n=12 non prothetic valve cardiopathy, n=21 treatment of thromboembolic disease.The average follow-up was similar in both groups (median of 6.5 months in group A vs.7.5 months in group B). There were few complications: 1 mild epistaxis and 1 thrombotic event in group B, and none in group A. There were no differences in the proportion of controls outside the target range between both groups; there was a moderate proportion of controls outside the target range of INR, with equal distribution above and below the range. The median dose used to achieve the target INR was 0.3 mg/kg/d in Group A and 0.4 mg/kg/d in Group B. *Conclusions.*Oral Anticoagulant therapy is safe and effective in pediatric patients, with very low rates of thrombotic or hemorragic complications including those undergoing the Fontan surgery.

0163

LOW DOSE ASPIRIN AND ENOXAPARIN IN ANTIPHOSPHOLIPID SYNDROME (APS) ASSOCIATED WITH RECURRENT PREGNANCY LOSS (RPL)

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Background. Pregnant women with APS associated with RPL treated with unfractionated heparin (UFH) and low dose aspirin (ASA) resulted in an 80% viable infant delivery (Am J Obstet Gynecol 1996:174:1584-9). *Aims.* To evaluate the efficacy and side effects of treatment with low molecular-weight heparin (enoxaparin) combined with low dose ASA in patients with positive antiphospholipid antibodies and RPL. *Methods.* Prospective, single-institution observational study. 31 patients (pts) were enrolled. Mean chronological age was 33.4 years. All pts had 2 or more first trimester losses and had a complete evaluation for RPL including anatomical, hormonal, infectious, chromosomal, and immunological. A standard ELISA was employed to detect the presence of IgG, IgM, and IgA serum antibodies against the phospholipids cardiolipin, inositol, serine, and ethanolamine. All pts received ASA 81 mg orally and enoxaparin 40 mg subcutaneously daily as soon as the serum pregnancy test became positive. Pts were monitored at close intervals. *Results.* Obstetrical outcome: Preterm 1 (3%); Term 26 (84%); Abortion 4 (13%). Bleeding episodes: Major 0; Minor (epistaxis) 3 (10%). Mode of delivery (n=27): Cesarean section (C/S) 12 (45%); Vaginal 15 (55%).Average blood loss at delivery: C/S 975 cc; Vaginal 550 cc. Epidural Hematomas: 0. Deep Venous Thrombosis: 0. Maternal Thrombocytopenia: 0. *Conclusions.* The use of low dose ASA combined with enoxaparin during pregnancy for the prevention of RPL in women with APS appears to be safe. When compared with historical controls it seems to be at least as effective as UFH and low dose ASA.

0164

THE IMPACT OF ANEMIA, C-REACTIVE PROTEIN AND MEAN PLATELET VOLUME ON THIRTY-DAY MORTALITY IN PATIENTS WITH ACUTE ST-SEGMENT ELEVATION MYOCARDIAL INFARCTION TREATED WITH PRIMARY ANGIOPLASTY

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Background. Anemia, C-reactive protein and mean platelet volume (an indicator of platelet activation) are independent predictors of adverse outcome in patients with acute myocardial infarction. *Aims.* Due to their interrelated pathophysiology, the purpose of the study was to examine them together in the clinical setting. *Methods.* The study included 543 patients (pts) with first ST-segment elevation myocardial infarction (STEMI), admitted to the University hospital from January 2001 to December 2007. In all the pts angioplasty of the culprit lesion was performed (only pts with <12 h after the onset of symptoms were included and pts with malignant or inflammatory disease were excluded from the study). Whole group characteristics: 62% man, mean age 65 years, diabetes in 27%, hypertension in 64%, current smoking in 35%, hyperlipidemia in 34%, infarct related artery: left anterior descending artery in 43%, left circumflex artery in 14%, right coronary artery in 43%, Killip class >1 in 14%, multivessel disease in 54%, TIMI flow: >1 pre PCI in 22%, >1 post PCI in 96%. Hematocrit (Hct), C-reactive protein (CRP) and mean platelet volume (MPV) were obtained at the time of admission and samples were processed within 1 h of venepuncture. The primary end point was all-cause mortality within 30 days after admission. *Results.* 41 pts (7.5%) died during the 30-day follow-up. Nonsurvivors (NS) were older, more frequently females and were more likely to have diabetes (P<0.01). Less smokers and pts with dyslipidemia were in NS group (P<0.01). Congestive heart failure, anterior location of the myocardial infarction and multivessel disease were more prevalent in NS group, as well as worse baseline and final TIMI flow (P<0.01), while time to treatment was comparable in both groups. CRP (24.7 mg/L vs 12.6 mg/L, P<0.001) and MPV (8.92 fL vs 8.54 fL, P=0.001) were higher, while Hct (38.8% vs 41%, P<0.001) was lower in NS. Platelet count did not differ between the groups (P=0.55). In the univariable analyses anemia (Hct <39% for men and <36% for women), elevated CRP (>6.7 mg/L) and MPV (>8.5 fL) (cutoff points of CRP and MPV were calculated with ROC analysis) were significantly associated with the primary outcome (P<0.01). After multivariable adjustment for gender, age, cardiovascular risk factors, Killip class>1, TIMI flow, multivessel disease, anterior infarction and the remaining two of the examined laboratory parameters, the odds ratios in prediction of 30-day mortality were: 3.67 (P=0.005), 2.94 (P=0.03) and 1.35 (P=0.40), for anemia, elevated CRP and MPV respectively. *Conclusions.* Anemia and CRP, but not MPV, are independently related to short term mortality in pts with STEMI treated with primary percutaneous coronary intervention.

0165

THE MAGELLAN STUDY METHODOLOGY: RIVAROXABAN COMPARED WITH ENOXAPARIN FOR THE PREVENTION OF VENOUS THROMBOEMBOLISM IN HOSPITALIZED MEDICALLY ILL PATIENTS

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Background. Results of recently completed trials comparing low molecular weight heparins (LMWH) or pentasacharride with placebo controls in medically ill patients have provided convincing evidence for venous thromboembolism (VTE) prevention. The efficacy and safety of VTE prevention with oral rivaroxaban 10 mg od for 35 days has been demonstrated in elective hip replacement patients. *Aims.* To compare treatment with oral rivaroxaban once daily for up to 39 days to short-

er term treatment with subcutaneous enoxaparin 40 mg once daily for up to 14 days and to compare short term rivaroxaban with short term enoxaparin both administered for up to 14 days for VTE prevention and safety in hospitalized medically ill patients. *Methods.* Study design: A multinational, multicenter, randomized, double-blind, double-dummy, active comparator controlled study in patients with current and likely ongoing reduced mobility. All enrolled patients receive study medication, undergo mandatory bilateral lower limb venous ultrasonography on Day 10±4 and on Day 35±4, and are followed for an additional 60 days. Symptomatic VTE is investigated at anytime. Inclusion criteria: Age ≥40 hospitalized with: heart failure, active cancer; or acute ischemic stroke with leg paresis. Or acute infection, acute inflammatory or rheumatic disorders or acute respiratory insufficiency, immobilized, and with at least one risk factor e.g: age >75 years, previous venous thromboembolism, previous cancer or heart failure, severe venous disease, thrombophilia, recent major surgery or serious trauma, hormone replacement therapy, morbid obesity (body mass index ≥35 kg/m^2). Exclusion criteria: Include an increased risk of bleeding; prohibited drugs or procedures: e.g anticoagulant therapy; concomitant conditions or diseases such as: allergies, severe renal or liver disease. Outcomes: The primary efficacy outcome is the composite of asymptomatic proximal DVT detected by mandatory compression ultrasonography, symptomatic proximal and distal DVT, symptomatic PE and fatal VTE reported during the treatment phase of the study: The primary safety outcome is the composite of major bleeding events and non-major clinically relevant bleeding events. All subjects have rivaroxaban levels measured at various time points. In selected centers, a full PK/PD profile is performed. Pharmacogenetics and health economic outcomes are also being assessed. Statistical *Methods.* Primary efficacy analysis: There are two efficacy analysis populations, pertaining to the two primary efficacy endpoints. The study is powered at the 90% level to show non-inferiority at Day 10±4 days and superiority at Day 35±4 days. ~8000 patients will be randomized from ~530 sites in 52 countries. The first patient was enrolled in December 2007. *Results.* Current status: 01 March 2010, ~7300 subjects have been enrolled into the study from ~530 actively recruiting centers in 52 countries. Mean age is ~70 years, ~46% are female, ~34% have heart failure, ~9% have active cancer. ~15% have acute ischemic stroke, ~37% have acute infectious and inflammatory diseases including acute rheumatic diseases and ~22% have acute respiratory insufficiency. *Summary/Conclusions.* The MAGELLAN study will determine the efficacy and safety of an extended duration of treatment with rivaroxaban compared to current standard of care.

0166

TREATMENT OF DEEP VEIN THROMBOSIS WITH CONTINUOUS IV INFUSION OF LMWH IN CHILDREN IN THE YEARS 2003 - JUNE 2009

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Backround. Incidence of the thrombosis is age dependent with the lowest risk in the childhood. Children mostly suffer from vein thrombosis. Occurrence of thrombosis in children is 0,07/10 000, but it is higher among hospitalized children (3,5/10 000). Low molecular weight heparin (LMWH) is preferred treatment of deep vein thrombosis (DVT) in children. The most frequent way of application of LMWH is via subcutaneous injection. The treatment with continuous iv infusion might be advantageous (no pain, probably lower risk of bleeding related to shorter half-life) especially in patient with permanent i.v. access. This approach was adopted from the treatment with unfractionated heparin. *Aims.* To prove efficacy and safety of the treatment of deep vein thrombosis with LMWH by continuous intravenous infusion in children in the pilot project. *Methods*. In this pilot retrospective study we present group of 32 children with DVT, who were treated with continuous infusion of LMWH for their first thrombosis from 2003 till June 2009. Our current data are an update of those presented on ASH 2006. There were 18 (56%) boys and 14 (44%) girls. Duration of the treatment with LMWH was modified in accordance with the course of thrombosis (monitored by Doppler ultrasound with compression). The median duration of the treatment was 13,5 days (ranging from 5 to 44 days). The dose of LMWH was adjusted according to antiXa levels. Required therapeutic range was 0,5-1 IU/mL. The median dose of LMWH was 240IU/kg/24h, with minimal dose 200 IU/kg/24h and maximal dose 330 IU/kg/24h. *Results.* The treatment with continuous infusion led to total recanalisation of the vein in 11 cases (34.4%), partial recanalisation was achieved in 16 (50%) patients. Only five (15.4%) patients were without any recanalisation. We have not noticed any bleeding as adverse event of the treatment in any of our patients. *Conclusion.* We would like to point out that the treatment of DVT with continuous infusion of LMWH in children might be efficient and safe alternative to SC application under certain circumstances especially in children with indwelling central venous lines or who are endangered by the risk of bleeding complications for example in children with thrombocytopenia. By means if this treatment we can avoid repeated painful subcutaneous injections and thus increased child's quality of life.

0167

COAGULATION AND FIBRINOLYSIS STATUS IN PATIENTS WITH MULTIPLE SCLEROSIS

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Backround. Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) of unknown etiology and is the most common neurologic entity in young adults. In general, the inhibition of the plasminogen activator system and the subsequent accumulation of fibrin have been associated with axonal damage in multiple sclerosis lesions.In an animal model of multiple sclerosis (experimental autoimmune encephalomyelitis), abnormalities in fibrinolytic state and fibrin deposition have been reported around CNS blood vessels. *Aims.* The objective of this study was to evaluate the levels of blood coagulation and fibrinolysis components of the hemostasis system in patients with multiple sclerosis. *Methods.* A total of 47 (36 females and 11 males, mean age 36.7±8.3 years) patients suffered from various clinical forms and at various stages of MS were enrolled to the study. Patients with co-existing liver, renal, hematological or other autoimmune diseases and those under anticoagulant treatment were excluded. Twenty (14 females and 6 males, mean age 42.4±6.7 years) healthy adults consisted the control group. Coagulation and fibrinolysis parameters were measured in plasma samples using an automated analyser (ACL Top, ALAPIS). Results were statistically analysed using Student's t test. *Results.* The laboratory parameters examined are shown in Table 1. D-dimer levels were significantly higher (P=0.04) in MS patients when compared to the control group. In female patients D-dimer levels were observed to be higher (249.78±121.29), but not significantly (P=0.34) in comparison to those of the male MS group (224.83±129.32). FVIII values were also significantly higher (P=0.007) in MS patients. FXII was also increased but not significantly important compared to the control group. Plasma PT, aPTT, fibrinogen, protein C, antithrombin-III and protein S activity, activated protein C resistance, lupus anticoagulant, coagulation factors V, VII, XI, plasminogen and α2-antiplasmin levels were not different between the two groups. *Summary/Conclusions.* The study shows a significant increase in FVIII levels and D-Dimer in patients with MS. The coagulation and fibrinolytic alterations should be further investigated and correlated with MS clinical forms, progression and treatment of the disease in order to achieve a better understanding of the role of the hemostasis system in the pathogenesis of MS.

Table 1. Coagulation and fibrinolysis parameters in patients with MS

Coagulation parameters	MS patients	Control patients	*p*-value
PT (sec)	11,13±0,82	11,19±0,60	0,76
INR	0,92±0,06	0,92±0,04	0,95
aPTT (sec)	27,94±4,06	29,45±2,67	0,14
Fibrinogen (mg/dl)	341,75±62,73	344,47±44,73	0,86
Protein C (%)	125,69±25,62	113,12±16,24	0,07
ATII (%)	110,9±16,35	103,26±15,82	0,09
Protein S (%)	94,24±14,70	100,47±18,20	0,19
APC-r Ratio	2,56±0,40	2,70±0,38	0,23
LA Ratio	1,06±0,11	1,06±0,06	0,84
FVIII (%)	162±67,13	112,88±29,40	0,007*
FXI (%)	156,95±49,18	138,71±38,13	0,38
FV (%)	115,53±30,33	115,67±25,48	0,99
FXII (%)	111,40±21,24	113,00±26,55	0,84
FVII (%)	100,05±23,46	103,13±19,30	0,74
Fibrinolysis parameters	**MS patients**	**Control patients**	***p*-value**
D-Dimer (ng/dl)	240,94±115,09	174,81±89,10	0,04*
Plasminogen (%)	103,00±17,00	99,50±8,83	0,62
α2-antiplasmin (%)	103,55±20,75	104,81±11,90	0,82

0168

INCIDENCE OF VENOUS THROMBOEMBOLISM IN PATIENTS WITH PRIMARY MEDIASTINAL LARGE B-CELL LYMPHOMA: A RETROSPECTIVE ANALYSIS OF 42 PATIENTS

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Background. Primary mediastinal large B-cell lymphoma (PMBCL) is a rare subtype of diffuse large B-cell lymphoma, arising in a mediastinum. Compression of large mediastinal vessels is common in patients with PMBCL, predisposing these patients to venous thrombosis. The incidence of this complication is still unknown. *Aim.* The aim of our retrospective study was to analyze the incidence and risk factors for venous thromboembolic disease (VTE) and its correlation with clinical and laboratory variables, as well as with survival in patients with PMBCL. *Patients and Methods.* We analysed 42 PMBCL pts who were treated at The Clinical Center of Serbia between June 1999 and November 2009. Evidence of VTE, the time to thrombotic event and risk factors to these trombotic events were sought. We compared age, gender, risk factors, laboratory variables (Hb, Le, Tr), hemostasis parameters (fibrinogen, PV, PTT, D-dimer), diameter of mediastinal tumor mass, incidence of syndrome venae cava superior (SVCS) and survival between two groups of PMBCL patients: patients with VTE (VTE group) and those without VTE (NVTE group). All patients with VTE received low molecular weight heparin (LMWH). The median follow up was 59 months. *Results.* In the VTE group there were 15/42 pts (36%) while 14 pts had deep venous thrombosis and 1 had pulmonary emboli. Mean age in VTE group was 38±15 (M/F=5/10) while in NVTE was 32±10 (M/F=8/19). Ten patients (24%) had a thrombosis at the moment of diagnosis PMBCL, while in five (12%) thrombosis occurred during the course of the disease. The majority of patients had the thrombosis of v. subclaviae and v. jugularis internae (10 pts), while 1 pts had thrombosis of v. cavae superior, 1 v. femoralis and 1 v. axillaris. The majority of patients in the VTE group had a risk factor which were distributed as follows: 9 pts (60%) were smokers (F/M=46%/54%); 2 (5%) had a history of surgery within the last 3 months; 3 (7%) had a pregnancy; 1 (2%) was taking the oral contraceptive pill; 1 (2%) had the previous history of VTE; 1 (2%) had the AFS and 1 (2%) was heterozygote for mutation FII 20210A. Leukocytes and platelets at diagnosis were not statistically different between patients in the VTE and the NVTE group. According to hemostasis variables, patients in the VTE group had significantly higher fibrinogen (P=0.02) and D-dimer (P=0.001). Also, patients in the VTE group had significantly larger diameter of mediastinal tumor mass (P=0.01) and the incidence of SVCS (P=0.009). The 4-yrs overall survival for the VTE group and NVTE group were 51% and 68,5% respectively (log rank P=0.058). *Conclusions.* Venous thromboembolism is a common complication in PMBCL patients, especially in those with large (bulky) mediastinal tumor mass. VTE could be the initial manifestation of PMBCL and according to our results negatively influences survival in PMBCL patients.

Clinical bleeding

0169

HYPERFIBRINOLYSIS DURING LIVER TRANSPLANTATION: CAN POINT-OF-CARE THROMBOELASTOGRAPHY REPLACE PROPHYLAXIS? COMPARING ANTIFIBRINOLYSIS AND VOLUME OF BLOOD PRODUCTS TRANSFUSED - ONE CENTER'S EXPERIENCE

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Background. Bleeding during liver transplantation (LT) can be due to the primary disease, peri-operatory complications, and hyperfibrinolysis (HF), and can be controlled with blood products (BP) - packed red blood cells (PRBC), platelet concentrate (PC) and fresh frozen plasma (FFP). Prophylaxis with aprotinin minimized bleeding due to HF until its approval was discontinued. Point-of-care rotational-thromboelastography (ROTEG) evaluates platelet function, coagulation and fibrinolysis, identifying bleeding due to HF, which can be specifically treated with antifibrinolytics. *Aim.* To compare the use of antifibrinolytics and BP during LT when prophylaxis was used, and after point-of-care ROTEG was introduced, to evaluate whether ROTEG-directed treatment offers equal results to prophylaxis. *Methods.* We reviewed 293 LT performed in our Hospital from January 2004 to December 2009. We excluded 64 pediatric transplants, 8 cases with incomplete charts, and 86 transplants for familial amyloid polyneuropathy (never subjected to prophylaxis in our center). *Results.* Patients who underwent Prophylaxis used 10.04±10.75 units of PBRC, compared to 25.20±16.87 for those without prophylaxis who were Treated for HF (P<0.001) and 11.07±16.88 for those without prophylaxis who were Untreated (P=NS). Use of FFP was 10.97±14.83 units in the Prophylaxis patients, 41.94±22.34 in the Treated (P<0.001) and 18.55±20.76 in the Untreated (P=NS). Use of PC was 7.88±7.93 units in the Prophylaxis patients, 15.35±9.37 in the Treated (P=0.006) and 5.60±8.11 (P=NS) in the Untreated. Of the patients who underwent point-of-care ROTEG, those who had HF and were treated (HF-Treated) used 27.57±17.57 units of PRBC, while those who were not (HF-Untreated) consumed 15.17±21.11 (P=NS); the use of FFP was 45.57±23.04 units in the HF-Treated patients and 24.83±26.96 in the HF-Untreated (P=0.049); the use of PC was 16.92±9.32 units in the HF-Treated patients and 6.75±8.01 in the HF-Untreated (P=0.006). In the HF-Treated group, the volume of PRBC administered before treatment was started was 21.64±16.87 units, of FFP was 34.64±21.69, and of PC was 8.71±5.40. Compared to patients undergoing prophylaxis, consumptions in those with ROTEG-confirmed spontaneous absence of HF were 8.78±13.06 units of PRBC (P=NS), 16.93±17.55 of FFP (P=NS) and 4.04±6.73 of PC (P=0.021). *Discussion and Conclusions.* We found that patients who underwent prophylaxis consumed BP on par with those who spontaneously never developed HF, while the use of BP was significantly higher in patients without prophylaxis, treated with antifibrinolytics. Considering patients without prophylaxis with ROTEG-confirmed HF, we identified two subsets - one with lower consumption of BP, not specifically treated for HF, and one with a high consumption of BP, treated for HF (after extensive transfusion). These results, coupled with the fact that antifibrinolysis in HF should not worsen bleeding, suggest that the patients who were treated were the ones who were showing uncontrolled hemorrhage. Treatment with antifibrinolytics after extensive bleeding has started is unsatisfactory, and offers worse results in terms of BP use than were obtained with prophylaxis with aprotinin. Point-of-care ROTEG could improve on the present results if antifibrinolysis is instituted as soon as ROTEG-evidence of HF is found, and not only after bleeding has started.

0170

TREATMENT OF ACENOCUMAROL-ASSOCIATED COAGULOPATHY WITH ORAL VITAMIN K

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Background. Optimal treatment for acenocumarol anticoagulated patients with a high INR is not well established as most studies have included warfarin treated patients. The few studies including acenocumarol over-anticoagulated patients come to the conclusion that vitamin K is not necessary and can be dangerous as most patients become under-anticoagulated. However the use of oral vitamin K in

these patients has become routine clinical practice in many acenocumarol clinics and hospitals. *Aims.* To determine the percentage of patients over-anticoagulated with acenocumarol achieving their therapeutic INR range the following day after administration of oral vitamin K individualised according to initial INR. To determine the impact of patient age, gender, weight, height, body surface area, acenocumarol daily dose, target INR and CYP2C9 and VKORC1 polymorphism on the rate of INR reversal. *Methods.* Patients on acenocumarol treatment with INR >5 asymptomatic o with mild bleeding were included. The day's dose of acenocumarol was omitted and oral vitamin K1 (phytomenadione) was administered according to the following diagram: none for INR <6, 1 mg for INR 6-9.9, 2 mg for INR 10-14.9, 3 mg for INR 15-19.9 and 4 mg for INR ≥20. INR was again determined the following day (day +1). Written informed consent was obtained. *Results.* Three hundred events (135M/165F) were included in the study. Total number of patients was 248, with 45 of them having more than one episode requiring vitamin K. Median age was 79 years (range 19-98). Initial INR ranged from 5.1 to 22.1 (median 6.1). In 157 events only omission of acenocumarol was required, while 143 events required vitamin K in addition (1 mg in 115 events, 2 mg in 20 events, 3 mg in 6 events and 4 mg in 2 events). Median INR at 24 hours was 2.49 (range 1.1-10.7). Out of the 300 analysed events, INR at day +1 was in range in 140 (46.7%) and in 226 (75.3%) it was within a safe range of INR 1.8 to 4.0. Within the group needing vitamin K, 28% of patients had INR <1,8 at day +1. *Conclusions.* Acenocumarol withdrawal and vitamin K administration according to initial INR lowers INR to a safe range in most patients. However treatment of patients whose INR is <1.8 at day +1 needs improvement by lowering vitamin K dose and/or by identifying other responsible factors. The influence of clinical factors and CYP2C9 and VKORC1 polymorphisms on the extent of INR reversal will be retrospectively determined. Authors are grateful to the medical staff at the Haematology Section of Sierrallana Hospital and the Health Centres in the Primary Health Care areas in Torrelavega-Reinosa for their help.

Table.

INR AT DAY +1	TARGET RANGE	INR<1.8	INR 1.8-4.0	INR>4.0
ALL EVENTS (n=300)	140 (46.7%)	43 (14.3%)	226 (75.3%)	31 (10.3%)
No vitamin K (n=157)	82 (52.2%)	3 (1.9%)	135 (86%)	19 (12.1%)
With vitamin K (n=143)	58 (40.6%)	40 (28%)	91 (63.6%)	12 (8.4%)

0171

CANCER-RELATED DISSEMINATED INTRAVASCULAR COAGULATION: FIRST LINE THERAPY WITH PLASMA-DERIVED PROTEIN C

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Background. Cancer-related disseminated intravascular coagulation (DIC) is a rare but life-treating condition. Acute management is mainly based on administration of fresh-frozen plasma (FFP) and Antithrombin (AT) with the aim of restoring coagulation factors deficiency. The Protein C (PC) pathway is a modulator of the coagulation as well as the inflammatory system; PC deficiency leads to increased activation of the coagulation system, resulting in thrombin generation. Some clinical trials have demonstrated the efficacy of administering activated recombinant (a-r) PC in adult patients with sepsis-related coagulopathy, but increasing the risk of major bleeding. Plasma-derived PC has a self-limiting process in determining anticoagulation thus it seems more suitable than a-rPC in patients at high risk for bleeding, such as cancer patients. *Aims.* To describe the efficacy and safety of PC concentrate to restore physiological values in adult cancer patients with overt DIC. Study Design. Not controlled clinical trial (NCCT). Materials and *Methods.* Adult cancer patients affected by DIC, having PC plasma concentration less than 50%, were treated with PC concentrate (Ceprotin®, Baxter) as an adjusted bolus of 30 to 50 UI/Kg/die to restore normal PC values (70-120%). Clinical outcomes (bleeding, thrombosis and mortality) were recorded up to a follow-up of 28 days from the initial diagnosis of DIC. PC activity, WBC, platelets, D-dimer, fibrinogen, PT, aPTT, AT and DIC score were measured after 12, 24, 48, 7 and 10 days. *Results.* Twenty-two patients were included over a period of 3 years; among them 16 had solid cancer and 6 had haematological cancer. All patients had advanced/metastatic neoplasm. PC concentrate normalized PC activity in all patients within 48h and remained upper the lower normal value for the following days. Baseline PC levels were lower in non-survivors than in survivors although this difference was non-significant. During the study period, there was a significant increase of platelets, fibrinogen, PT, AT, and a significant decrease of D-dimer, aPTT and DIC score (Table 1). No bleeding or thrombosis were observed; mortality at 28 days was 35%. *Conclusions.* Our investigation shows that PC concentrate is safe and normalizes laboratory variables in cancer patients with overt DIC.

Table. Changes in laboratory findings obtained from all patients during the study period (mean±SD)

	Baseline	24 h	48 h	72 h	7th day	14th day
PC (%)	27.3 ± 7.1	71 ± 15.6*	85.9 ± 12.5*	91.2 ± 11.6*	92.2 ± 13.4*	99.1 ± 13.5*
WBC ($\times 10^9$/L)	8.2 ± 3.1	7.8 ± 2.2	6.5 ± 1.9	6.7 ± 1.5	7.3 ± 1.5	8.1 ± 0.6
Platelet ($\times 10^9$/L)	49.3 ± 20.4	51.2 ± 19.4	71.2 ± 33.4	91.7 ± 41.1	113.4 ± 65.1	154.8 ± 109.2*
D-dimer (µg/L)	2.133.6 ± 1.643	2.366 ± 1.561	1.230 ± 1.045*	800.2 ± 686*	350 ± 225*	541 ± 246*
Fibrinogen (g/L)	2.1 ± 1.4	2.8 ± 1.1	3.6 ± 1.5	4.4 ± 1.4*	4.5 ± 1.2*	4.2 ± 1.3
PT (%)	46.4 ± 11.5	46.2 ± 12.1	51.8 ± 13.8	63.3 ± 15.2	65.4 ± 0.9*	69.7 ± 14.3*
aPTT (s)	40.1 ± 13.4	34.8 ± 7.6	35.4 ± 6.1	33.4 ± 6.1	32.9 ± 7.5	31.2 ± 3.6*
AT (%)	54.2 ± 12.2	61.6 ± 23.3	73.4 ± 21.4	77.7 ± 22.2*	80.6 ± 16.5*	87.1 ± 18.5*
DIC score	6.26 ± 1.12	5.38 ± 1.42	4.26 ± 0.96	3.16 ± 0.98*	2.97 ± 0.87*	2.21 ± 1.43*

*P < 0.05 versus baseline

0172

ORTHOPEDIC SURGERIES IN SEVERE HEMOPHILIA PATIENTS ON A SHOE STRING BUDGET

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Background. Management of severe hemophilia patients, in resource poor developing countries remains a major challenge to the care givers. The problems are further compounded in patients requiring surgeries. Thus, even orthopedic surgeries in hemophilia patients require much more planning and effort than in non hemophilics. Several studies have shown that osteoporosis is a universal phenomenon in hemophiliacs' all over the world. The magnitude of this osteoporosis is much more in developing countries and hence the prevalence of complications and fractures of the long bones is also much higher. *Methods.* Over the last 10 years, our Comprehensive Hemophilia Care Center has carried out various orthopedic surgeries in 20 hemophilia patients with fractures and pseudotumors, age range from 8 - 45 years. Of these 6 had hemophilia B (5 severe, 1 moderate), 14 had hemophilia A (1 moderate, 13 severe) and 3 hemophilia A patients had inhibitors. The factor concentrates used for the surgeries were optimized, from our previous experience, along with generous use of anti fibrinolytic drugs, oral, parenteral and local. *Results.* The factor concentrates used in the 20 surgeries ranged from 5000-32,000 units, (mean 12039.47±21.09 units SEM). 2 of the patients with inhibitors received modest doses of FEIBA (3000 - 7000 units) and 1 received FEIBA and factor VIIa. All 3 patients also received parenteral anti fibrinolytic drugs. 2 patients developed inhibitors postoperatively and had significant bleeding, requiring blood transfusions. 1 patient without inhibitors had moderately severe bleed and wound infection, needing prolonged blood product and antibiotic support. Factor support was given only for 7 - 10 days in all patients; most of the patients received anti fibrinolytic drugs. All the patients were discharged with good functional activity, without any mortality or residual morbidity due to the surgery. *Summary/Conclusions.* The present study clearly shows that major orthopedic surgery in patients with severe hemophilia can be accomplished with 30-35% of the total amount of factor concentrates which is utilized in the developed countries, without compromising on the outcome. This has been possible by reducing the amount of factor, accepting a lower trough level, intra and post operative use of anti fibrinolytic drugs. The use of FEIBA works out too expensive in our inhibitor patients; thus by adding

adjuvant anti fibrinolytic drugs, we have been able to cut down the requirement of FEIBA from 75-100 units/kg/dose, to as little as 25 units/kg/dose without increasing the inherent thrombotic risk of this combination and still achieving good hemostasis. This has major implications for hemophilia care not only in developing countries but also in the developed countries where the health systems are strained due to the very high cost of lifelong hemophilia care.

0173

A FAVORABLE OUTCOME OF DISSEMINATED INTRAVASCULAR COAGULATION IN CHILDREN USING RECOMBINANT THROMBOMODULIN

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Background. Disseminated intravascular coagulation (DIC) is a serious complication that can occur during treatment of hematological malignancies and severe infection. Conventional treatment of DIC consists of heparin administration as well as platelet transfusion or fresh frozen plasma (FFP) supplementation. Recombinant thrombomodulin (rTM) has been developed as a new-type treatment that inhibits Factors Va and VIIIa by activating protein C. A phase III randomized study demonstrated the superiority of rTM to heparin; however, pediatric patients were not included in this study and no data for children with DIC is currently available. From July 2009, we therefore began treatment of 10 children with DIC using rTM.Aim To elucidate the efficacy and safety of rTM against DIC by retrospectively analyzing the outcome in 10 children with DIC (5 boys and 5 girls, age ranging from 1 day to 13 years old). *Methods*. Diagnosis of DIC was made according to the diagnostic criteria of the Japanese Ministry of Health and Welfare. rTM (130 U/kg/day for 2 newborns and 380 U/kg for the remaining 8 patients) was administered daily. No other anti-DIC agent except flesh frozen plasma and platelet transfusion was used concomitantly. *Results*. The causes of DIC were hematological disorders (n=6) and severe infection (n=4). A coagulation test at diagnosis showed the following abnormalities: median platelet count of 31×10^3/L, median prothrombin time (PT) ratio of 1.5 (normal: 0.9-1.1), median fibrin degradation products (FDP) level of 16.4 mg/L (normal: <4.0 mg/L), median D-dimer level of 11.4 mg/L (normal: <1 mg/L), median ATIII level of 84% (normal: 70-130), and median fibrinogen level of 3.54 g/L (normal: 1.5-4.0). rTM was used for a median of 5 days, ranging from 3 to 13 days. After 6 days of administration, laboratory data improved as follows (median): PT ratio, FDP, D-dimer, ATIII and fibrinogen levels were 1.28, 7.0 mg/L, 2.7 mg/L, 111% and 3.6 g/L, respectively. DIC was resolved at 3-5 days after administration of rTM in 7 patients. In contrast, the 2 newborns (diagnosed with Down's syndrome-associated transient myeloproliferative disorder and acute lymphoblastic leukemia with mixed lineage leukemia gene rearrangement, respectively) failed to recover from DIC. In both patients, pulmonary bleeding and intracranial bleeding occurred during or after rTM treatment, and they died of liver dysfunction and multiple organ failure at 19 and 21 days old, respectively. The remaining 8 patients were well at 2-7 months after the diagnosis of DIC. *Conclusion.* rTM was highly effective in 7 out of the 10 children with DIC examined, resolving the DIC within 5 days. However, the outcome in the 2 newborns examined was unfavorable. Both patients presented a persistently low level of fibrinogen due to severe liver failure despite daily supplementation of FFP. Our treatment with rTM, FFP and platelet transfusion was suboptimal for these 2 patients. A more effective treatment strategy to prevent life-threatening bleeding must therefore be explored in patients with hepatic dysfunction. Overall, however, the current results are encouraging for children with severe DIC. Nevertheless, since rTM has so far been licensed only in Japan, more clinical investigations and a prospective study are necessary to clarify the distinct effectiveness of this novel drug.

0174

PLATELET FUNCTION AND COAGULATION ABNORMALITIES IN TYPE 1 GAUCHER PATIENTS: EFFECTS OF ENZYME REPLACEMENT THERAPY

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Background. Coagulation abnormalities and platelet disfunction were reported in Gaucher patients (GP) and might attribute to bleeding tendency. Data concerning the effects of enzyme replacement therapy (ERT) on coagulation abnormalities and platelet function are few and controversial. *Aim.* To investigate the impact of ERT (Cerezyme®) on bleeding tendency and haemostatic parameters of GP. *Methods.* 31 Serbian treatment-naive type 1 GP (M/F 17/14; median age 49 years, splenectomized 9/31) were studied. Complete blood count, protrombin time (PT), activated partial tromboplastin time (aPTT) and coagulation factors activities were measured according to standard *Methods.* Platelet aggregation (PA) was assessed by a whole-blood aggregometer with collagen 10 μmol/L (Col10) and 5 μmol/L (Col5), adenosine5-diphosphate 10 μmol/L (ADP10) and 5 μmol/L (ADP5), ristocetin (Ris) 1.25 mg/mL, epinephrine (EPI) 10 μmol/L and arachnoid acid (AA) 2 mmol/L (normal PA≥60% for all inducers). Spleen volumes (SV) were assessed by CT and declared in MN ratio (MN = measured volume/normal volume). 21/31 patients were treated with Cerezime®. Bleeding tendency and haemostatic parameters were assessed after 6, 12 and 24 months of ERT (ERT6,12,24). *Results.* Pretreatment: Bleeding episodes (2 severe) were registered in 10/31GP. Mean platelet count (PC) was 150×10^3/L (range: 46-428); 22/31 GP had PC<150×10^3/L. PT (median 61%, range 43-72) and aPTT (median 41.45s, range 35.20-56.70) values were abnormal in 16/31 and 13/31 GP, respectively. The most frequent clotting factor deficiency was of FV (9/31). PA abnormalities were registered in 19/31 GP; 6/19 GP had reduced response to ≥2 inducers. PA was reduced in response to EPI (7/19 GP, mean 0.57) and AA (7/19 GP, mean 0.58). Mean PA values were normal in response to ADP5, ADP10, Col5, Col10 and Ris but the abnormal PA was registered in 19/19, 4/19, 13/19, 6/19 and 3/19 GP, respectively. SV inversely correlated with PC and reduced response to Col5, AA and ADP5 (P<0.05) Bleeding GP had significantly lower PC, higher chitotriosidase level and greater SV comparing to the non bleeding (P<0.01). After ERT: N° of bleeding GP significantly decreased after ERT6 (1/10 GP; P<0.01) and desapeared at ERT24. PC significantly increased at ERT6 and remained in normal range till ERT24 (Plt6 240×10^3/L, Plt12 240×10^3/L, Plt24 295×10^3/L; P<0.01). At ERT24 5/21 GP were still trombocytopenic. PT increased significantly from ERT0 to ERT24 (PT0 75%, PT6 73%, PT12 77%, PT24 85%; P<0.01). vWF increased significantly at ERT6 and ERT24 (vWF0 65%, vWF6 68%, vWF12 65%, vWF24 87%; P<0.01). N° of GP with abnormal PA decreased significantly at ERT6 (5/19; P<0.05). PA on AA and EPI normalized at ERT6: 0.76 and 072 respectively. At ERT24 the abnormal PT, APTT, vWF and PA values were still registered in 8, 1, 1 and 3 GP, respectively. Chitotriosidase level and SV significantly decreased acheaving: Chito24 1872 nmol/L P<0.05, spleen MN24 9, P<0.05. *Conclusions.* Bleeding together with decreased levels of coagulation factors and abnormal PA were registered in a considerable number of our GP. Cerezyme significantly decreased bleeding tendency and increased PC, PA and PT. Our data suggest that spleen and total body burden of glucocerebroside may contribute to the platelet disfunction and bleeding.

0175

INCREASED BONE METABOLIC TURNOVER IN PATIENTS WITH HEMOPHILIA

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Osteoporosis, as a clinical issue in patients with haemophilia, is rather recently recognized. Both disorders may be accompanied with chronic pain, progressive invalidity, loss of independence and increased mortality. We performed osteodensitometry using dual X-ray absorptiometry (DXA) in 12 adult patients with haemophilia A or B (median age 42 years), aiming to assess the incidence and severity of osteoporosis. Also, parameters of metabolic activity of the bone were measured (serum levels of osteocalcin, beta-cross laps, vitamin D (25 OH-D), calcium and parathyroid hormone /PTH/). We compared those results with result of a control group. Age and sex matched control group were patients from other clinics having risk factors for osteoporosis, who underwent DXA. Parameters of bone metabolic activity were compared to dataset from central laboratory, originating from age and sex matched group of patients from other clinics of our Clinical centre. Statistical tests performed were: t-test and other tests of descriptive statistic. Using WHO criteria based on values of bone mineral density (BMD) and Z score, 1 patient with haemophila had osteopenia (8.3%) and 2 had osteoporosis (16.6%). There were no statistical significance comparing lumbal and femoral BMD and Z scores from patients with

haemophilia and control group. But, some values of bone metabolic activity parameters were significantly higher in patients with haemophilia then in control group-osteocalcin was elevated in 4 patients (33.3%) (Median 40,5 vs. 26,8 ng/mL, P<0.05) and beta-cross laps in 8 patients (66.6%) (Median 771.3 vs. 305.3 pg/mL P<0.05). Other parameters (vitamin D, PTH and calcium) did not differ significantly. Osteoporosis seems to be frequent in patients with haemophilia, since a quarter of our patients had decreased bone mineral density. Lack of significant differences in DXA parameters between patients with haemophilia and those with known high risk for osteoporosis (control group), suggest that haemophilia itself is a risk factor for osteoporosis. Although, blood samples for measuring bone metabolic activity in patients with haemophilia were taken few hours or days after bleeding in joints or soft tissues, we suggest that our patients had highly increased metabolic turnover of the bone. We are aware that many factors could influence on the results of bone metabolic activity (comorbidity, age, life habits etc.), but we consider that our results raise a question about supplementation with vitamin D and calcium recently after bleeding episodes in patients with haemophilia.

0176

INHERITED BLEEDING DISORDERS IN THE PEDIATRIC AGE GROUP: SINGLE CENTER EXPERIENCE

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Objectives: The present study aimed to assess the prevalence and clinical presentation of inherited bleeding disorders in the Pediatric Hematology Unit, Children's Hospital, Ain Shams University, Cairo,Egypt. *Methods.* The hospital based registry was used to analyze the data of patients presenting with bleeding disorders along a period of 14 years starting from January 1st 1994 to December 31st 2007. The tests performed for diagnosis included mainly CBC, bleeding time (BT), platelet count, prothrombin time (PT), activated partial thromboplatin time (aPTT) and platelet Aggregation with ADP and ristocetin. , and bone marrow (BM) aspiration whenever necessary. *Results.* 687 patients with bleeding disorders were identified among 2949 hematology patients diagnosed in the Pediatric Hematology Unit in 14 years .Inherited defects of coagulation were observed in 187 (27.2%) of patients with bleeding disorders; hemophilia A was diagnosed in 132 patients (70.6%), hemophilia B in 26 patients (13.9%), factor I deficiency in 4 patients (2.1%), factor V deficiency in 3 patients (1.6%), factor X deficiency in 8 patients (4.2%), factor VII deficiency in 5 patiens(2.6%) , factor XIII deficiency in 2 patients(1.1%) , combined factor deficiency in 4 patients (2.1%) , and unclassified coagulation disorders in 3 cases (1.6%).Among 500 patients with platelet disorders immune thrombocytopenia was most common (74.8%), Glanzman's thrombasthenia was diagnosed in 56 patients (11.2%), Von Willebrands disease in 33 patients (6.6%), Bernard Soulier Syndrome in 5 patients (1%) and Chediak Higashi Syndrome in 2 patients (0.4%). In 30 patients (6%), the platelet function defect could not be classified into any specific subtypes.Most cases of coagulation disorders were diagnosed in the age group < 1 year (26.7%) and the median age was 33 months, while most cases of platelet disorders were diagnosed in the age group 6-<10 years and the median age was 72 months.In the present study 413 cases were males and 274 cases were females. All cases of Hemophilia A and B were male cases, while (30.8%) of other coagulation factors deficiencies and all unclassified were females. In inherited platelet disorders 52.6% were females . The presenting symptoms of coagulation disorders were in descending order: (25.1%) post circumcision bleeding, (22.5%) ecchymosis, (20.9%) hemoarthrosis and (15%) epistaxis.Among the presenting symptoms of rare coagulation disorders (other than hemophilia A and B), the most common symptoms were postcircumcision bleeding (20%)and bleeding umbilical stump (20%), followed by epistaxis (12%), hemoarthrosis (8%) and hematomas (4%). In our study , the presenting symptoms in rare inherited platelet disorders were as follows: purpura in(45.6%) , ecchymosis in (43.4%), epistaxis (20.4%) and bleeding gums (11.8%).Conclusion :The clinical expression of the inherited bleeding disorders is variable and may present challenges in both diagnosis and management. The awareness of the increased risk of these disorders will lead to higher index of suspicion and thus earlier diagnosis of severely affected infants who are at risk of serious bleeding.Centralisation and processing of data in reference centres is mandatory to increase knowledge on these uncommonand complex disorders, with the purpose of improving the management of these particular patients.

0177

IMPACT OF VON WILLEBRAND DISEASE ON HEALTH RELATED QUALITY OF LIFE IN A PEDIATRIC POPULATION

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Von Willebrand Disease (VWD) is the most frequent inherited bleeding disorder, whether VWD affects quality of life in children is unknown. This study determined health-related quality of life (HR-QoL) in children with VWD. All Dutch children known with moderate or severe VWD (defined as VWF:Ag or VWF:RCo ≤ 30%) were asked to participate. In total 133 children, 78 boys and 55 girls, aged 0-15 years participated. HR-QoL was assessed using the Infant and Toddler Quality of Life Questionnaire (ITQOL) in the age group 0-5 years, and the Child Health Questionnaire (CHQ) parent form for children aged 6-15 years. Bleeding severity was measured using the Tosetto Bleeding Score (TBS). In young children two domains showed lowered scores compared to normative data: general health (P<0.001), and parental impact time (P<0.001). Older children had lower scores than the normative data for four domains: physical functioning (P<0.001), role functioning emotional-behavioural (P=0.027), general health (P<0.001), physical summary (P<0.001). Type of VWD did not influence HR-QoL in young children. In older children different scores were observed between type 1 and 3 for six domains, and type 2 and 3 for three domains with a lower score for type 3 patients. The quartile of children aged 6-15 years with the highest TBS had lower HR-QoL in 9 domains in comparison to the quartile of children with the lowest TBS. Children with VWD have lower HR-QoL scores than children of the general population. In older children HR-QoL is associated with both type of VWD and TBS.

0178

CIRCUMSTANCES OF DIAGNOSIS AND CLINICAL FEATURES IN MILD/MODERATE FORMS OF INHERITED RARE BLEEDING DISORDERS AND OF VON WILLEBRAND DISEASE OBSERVED IN HAEMOPHILIA CENTRE OF PAVIA. ITALY

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Background. The haemorrhagic manifestations in mild/moderate forms of Rare Bleeding Disorders (RBD) and of von Willebrand disease (vWd) usually do not present clinical impact; furthermore screening clotting times may be normal or only slightly prolonged so that a variability of occasions induces to make a diagnosis. *Aim of the study*. To evaluate the circumstances of diagnosis and the bleeding patterns in mild/moderate RBD and vWd observed in the Centre of Haemophilia and other Congenital Coagulopathies of Pavia. *Patients*. 77 subjects (43 women) aged between 6 and 78 yrs; 33 with RBD including deficiencies of F XI (13), F VII (11), F X (2), F V (3), ipodysfibrinogenemia (2), F X+FXII (1), F VIII+FVII (1); 44 with vWd. *Results.* Circumstances of diagnosis: RBD Familiarity 40%, Abnormal clotting test 25%, cutaneous-mucosal haemorrhages* 20%, post-surgical bleeding 15%; vWd cutaneous-mucosal haemorrhages* 34%; familiarity 29%, post-surgical bleeding 23%, abnormal clotting test 14% (*including menorrhagia). Pattern of bleeding: In both RBD and vWd menorrhagia was present in 80% of women and post-partum haemorrhages in 7% of women with RBD and in 12% of women with vWd. In all patients: cutaneous-mucosal haemorrhages (%) RBD 40, vWd 66; post-surgical bleeding (%) RBD 20, vWd 30; deep haematomas (%) RBD 5, vWd 10.Haemarthroses were observed in one subject with F XI deficiency and in one subject with vWd. The bleeding manifestations were gen-

erally mild to moderate requiring RBC infusions in only seven patients, whereas in menorrhagia iron supplementation was frequently indicated. *Summary/Conclusions.* The main clinical manifestations of moderate/mild forms of RBD and vWd are cutaneous-mucosal haemorrhages and, in women, menorrhagia with occurrence in some subjects of postpartum haemorrhages. In RBD the diagnosis is frequently made because of familiarity or of abnormal clotting tests. In mild/moderate forms excessive post-surgical bleeding represents an occasion of diagnosis in about 1:5 patient with vWd and about 1:7 patient with RBD.

0179

DENGUE HEMORRHAGIC FEVER IN HEMOPHILIC PATIENTS: AGGRAVATION OF BLEEDING RISK

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Background. Dengue virus infection is commonly found in tropical countries. Pediatric patients including hemophiliacs are risk to be infected. The clinical manifestations of dengue virus infection vary from asymptomatic to mild degree of flu-like symptom of dengue fever and severe degree of dengue hemorrhagic fever (DHF). *Material and Methods.* Six moderate to severe hemophilia patients (4 hemophilia A, 2 hemophilia B) with the age ranging from 5 to 16 years of age were included in the study. The inversion of intron 22 was found in four hemophilia A patients. All of them presented with high fever, lethargy, loss of appetite, and bleeding manifestations at skin, epistaxis, gastrointestinal tract and hemothorax with the presumptive clinical diagnosis of DHF according to the criteria of World Health Organization. *Results.* The severity of DHF was graded according to the World Health Organization as follow: grade 1 with only positive tourniquet test (n=1), grade 2 with bleeding manifestation (n=1), grade 3 with threatening shock and massive bleeding (n=2) and grade 4 with profound shock and massive bleeding (n=2). All patients had their serological confirmation of dengue virus infection .They received appropriated fluid therapy to maintain the effective circulation during the febrile stage of leakage from increased vascular permeability and adequate blood component therapy of platelet concentrate, fresh frozen plasma, cryoprecipitate and factor concentrate. In addition, appropriate airway management, effective cardiovascular circulation, and infection control including hospital-acquired bacterial and fungal infection should be comprehensively provided. Dengue virus-associated hemophagocytic syndrome should be looked for and treated accordingly. *Conclusions.* Dengue virus infection is one of the serious complication among hemophilic patients in tropical countries. Aparted from vascular leakage, severe bleeding manifestation is one of the serious adverse event to unfavorable outcome.

0180

EPIDEMIOLOGY OF HEMOPHILIA IN MARTINIQUE (FRENCH WEST INDIES)

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Background. Hemophilia is a common form of hereditary bleeding disorder wide-spread in the world. It has an X-linked genetic transmission. Hemophilia A is due to deficiency of factor VIII and hemophilia B is due to deficiency of factor IX. Martinique is an island in the Caribbean, with a population consisting of 400 000 habitants. The half-breed population is mainly of Afro-Caribbean descendents. *Aims.* Census of hemophiliacs in Martinique. We have analyzed the type of hemophilia, the severity of the disease, the genetic mutations and the serology of hepatitis A, B, C, HIV, HTLV1; the presentation of factor VIII antibodies and the reasons of death. *Methods.* Census of hemophiliacs for the period 2000-2007. Data was taken from the medical charts at the Regional center for the treatment of hemophilia in Martinique and the interviews with the families. *Results.* A total of 76 patients with hemophilia was included, 67(88.2%) patients with hemophilia A and 9 (11.8%) patients with hemophilia B, of these 9(11.8%) with severe hemophilia, 5(6.8%) with moderate hemophilia and 62(81.6%) patients with hemophilia minor. Average age at the diagnosis: 2 years old for the cases of severe hemophilia, 16.5 years old for the mild hemophilia. Circumstances of discovery of the disease: haemorrhages 26 patients, blood tests 29 patients, family history 21 patients. Serological status: HAV 12 (15.7%), HBV 9 (11.8%) all born before 1980, HCV 17 (22.3%) born before 1987, HIV 7 (9.2%) all of them co infected with HCV and born before 1985, HTLV none. Factor VIII antibodies inhibitors: 2 patients (2.6%). Number of deaths: 3 (3.9%). Reasons of death: cerebromeningeal haemorrhage 1, HIV cardiomyopathy 1, HCV hepatocellular carcinoma 1. The principal genetic abnormalities for severe hemophilia A: inversion of the intron 22, deletion of the exons 1-6, deletion C6703 in the exon 25, for moderate hemophilia A: Cys 554 Phe, for minor hemophilia A: Leu 2053 Phe and Met 2255 Val, and for severe hemophilia B: point mutation, for minor hemophilia B: Phe 299 Val. *Conclusions.* The haemophiliac population of Martinique had the same epidemiological features when compared with the data from a Caucasian population. Contrary to the data in the literature concerning severe haemophiliacs, of African origin, the rate of inhibitors in our population wasn't superior. The genetic mutations were common for the majority of mutations. 5 mutations were not described.

0181

INHIBITORS AND LUPUS ANTICOAGULANT IN CONGENITAL HAEMOPHILIA

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Background. Patients with haemophilia can develop alloantibodies (inhibitors), as serious complication of replacement therapy, or autoantibodies which change coagulation, or both of them. *Aim.* This work describes two haemophilic patients with specific inhibitors and lupus anticoagulant (LA). *Results.* A 19 years old male patient with severe Haemophilia A and previously detected inhibitor and HCV infection developed intracranial hemorrhages. He was treated with rFVIIa 90 μg/kg/2h. Because very bad venous access he was prepared for central venous catheter (CVC) with 120 mg/kg/2h of rFVIIa. Four hours after implementation of CVC, severe neck oedema and breathless were developed and FEIBA was added at dosage of 80 U/kg. CT scan of the neck is shown diffuse large hematoma in muscle and soft tissue without mediastinal hemorrhage. Angiography confirmed that catheter was well implemented. Surgical exploration was shown that CVC has been placed through the muscle. Hemorrhage in the muscle produced severe compartment syndrome of the neck. Catheter was removed without bleeding. During second postoperative day severe compartment syndrome on the neck was dramatically evolved. Sutures have been removed immediately and respiratory function was improved. Control APTT was near normal (42.7s), and treatment was continued with concentrate of hFVIII in dosage of 4.000 U every 8h, during next 7 days, without bleeding. During the treatment with concentrate of FVIII levels of FVIII were in normal range. On day 14, APTT prolonged with increment of FVIII inhibitors. The treatment with hFVIII concentrate was stopped and rFVIIa continued. Second patient is 50 years old male with mild Haemophilia B and HCV infection who had time-depending inhibitors, and FIX inhibitors of 32 BU was detected. Plasma dilution test shown that higher dilution correlated with increase of FIX at it was corresponded with LA. LA 1.5 was detected by DRRVT. Before tooth extraction patient received 1800 U of FIX concentrate and one hour later APTT was prolonged but FIX was increased from 9% to 28%. Patient did not bleed after tooth extraction. It is well known that haemopilia, inhibitor, and LA prolonged APTT but it is not possible to be measured contribution each of them. Measurement of APTT in PRP before and after frozen and thaw in water bath on 37°C, shown decrease APTT from 90.7s to 72.9 s (Ratio=1.25) but inhibitors of FVIII were similar 5.6 BU and 5.4 BU respectively. Repeated test, when patient had FVIII inhibitors 13 BU were shown that APTT of PRP, after frozen and thaw on 37°C, shortened from 115s to 85.9s (Ratio=1.34). After frozen and thaw, platelets released phospholipids which neutralised LA and shortened APTT on the level which coresponded to haemophilia with inhibitors. *Conclusions.* It is well known that haemopilia, inhibitor and LA prolonged APTT but it is not possible to be measured contribution each of them. Usage of more than one test increase the chance of detecting LA and it may be helpful to differentiate LA from specific inhibitors. Differentiation of LA from FVIII inhibitors is crucial for different therapeutic management.

0182

CENTRAL NERVOUS SYSTEM BLEEDING IN RARE BLEEDING DISORDERS: SINGLE CENTER EXPERIENCE

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Rare bleeding disorders (RBDs) are autosomal recessively inherited disorders. Their frequency are approximately 1:500 000 to 1:2 000 000 in general population. Central nervous system is the most important site of severe bleeding in these patients. We assessed 109 patients retrospectively, who were diagnosed with RBDs (fibrinogen, FII, FV, FVII, FX, FXI, FXIII deficiencies) between 1990 and 2010. 69 patients were male and 40 were female. Their ages were ranging from one week to 19 years. Familial consanguinity was present in 38.2% of the patients 37.6% of these patients were asymptomatic. They were diagnosed by family histories or preoperative laboratory studies. Of these 109 patients 10 of them were diagnosed with fibrinogen deficiency, 5 with Factor V deficiency, 73 with VII deficiency, 11 with Factor X deficiency, 7 with Factor XI deficiency, 2 with Factor XIII deficiency. Easy bruising and skin bleeding 66.8%, hemarthroses 22.7%, oral cavity bleeding 21.2%, central nervous system bleeding 17.4%, epistaxis 17.4% and gastrointestinal bleeding 9.6% were the most common bleeding sites in our group. These 14 central nervous system bleeding cases were diagnosed as FVII deficiency (n:8, 57.1%), FX deficiency (n:4, 28.6%), FV deficiency (n:1) and fibrinogen deficiency (n:1). The presenting age of patients with central nervous system hemorrhages were in the first 3 months (71.5%). Since in our country consanguinity marriage is still a problem, hemorrhagic disorders in early month of life may be due to rare factor deficiency. In this study we aimed to emphasize early diagnose and prophylaxis of these disorders.

Clinical platelets

0183

CHRONIC IDIOPATIC THROMBOCYTOPENIC PURPURA (ITP) IN ADULTS - RETROSPECTIVE STUDY - SINGLE CENTER EXPERIENCE

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Background. The investigation and management of patients with chronic adult ITP varies widely and there is a lack of data on current treatment strategies in Europe. *Aims.* To contribute to a better understanding of the treatment and healthcare resource utilization of chronic ITP patients in Romania. *Methods.* This is part of a comprehensive project investigating the burden of chronic ITP and which involves both expert opinion as well as real patient data. A retrospective study for 350 ITP patients was performed. Patients' demographics, medical history, current treatments and side effects, as well as medical resource utilization were abstracted from the patient's medical charts for the 12 months prior to their most recent visit. *Results.* The mean age of the total patient sample was 49,1 years with 59% women and 41% men. Median time from the first diagnosis of ITP to the start of the observational period was 27 months. Prior to the observational period, 35% of patients had been splenectomized and the most frequently reported treatment was corticosteroids. During the observational period, 72% of all patients were treated and received 1-9 different medical treatments (mean 2.5). The most frequent reasons given for treatment were platelet count (72%), followed by bleeding symptoms (35%). Corticosteroids represented 71% of treatments given, followed by IVIg (13%), azathioprine (5%) and rituximab. Splenectomies (6% of patients) and platelet transfusions (2% of patients) were rarely performed during the observational period. 2% patients refused to undergo splenectomy during the period. In addition to regular monitoring of platelet levels, 81% of patients visited their hematologist 1 to 6 times during the year of observation. Main reasons for a visit were a low platelet count (48% of visits) and bleeding (19% of visits). Overall, 35% of patients required hospitalization; 8% of these were in an intensive care unit. 89% of hospitalizations were due to ITP (low platelet count, 69%, bleeding 31%) and 7% of hospitalizations were due to ITP treatment related side effects. Mean duration of hospitalization was 10,3 days. *Conclusions.* The retrospective chart review of 350 patients is the largest study to date of its kind in ITP and provides the first results of actual treatment practices, outcomes and medical resource utilization in our country. It showed that bleeding symptoms remained quite frequent among patients with chronic ITP even through two thirds of them were actively treated. Corticosteroids were the most widely used treatment, followed by IVIg.

0184

SAFETY ANALYSIS OF LONG-TERM ROMIPLOSTIM USE IN PATIENTS WITH CHRONIC IMMUNE THROMBOCYTOPENIA (ITP)

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Background. ITP is characterized by low platelet counts and increased risk of bleeding. Romiplostim is an Fc-peptide fusion protein (peptibody) that increases platelet production by binding to and activating the thrombopoietin (TPO) receptor, and is approved for the treatment of adult chronic ITP. We conducted a safety analysis of data pooled from all ITP romiplostim clinical studies. *Aim.* To evaluate the safety of long-term romiplostim treatment using the largest available set of clinical data. *Methods.* Data from 13 romiplostim clinical studies (patients treated from October 2003 to June 2009) in patients with ITP were analyzed. Patients received romiplostim, placebo, or standard of care (SOC); data from placebo/SOC patients were pooled. Given that the study time for patients while receiving romiplostim or placebo/SOC was not equal, adverse events were adjusted for study duration and reported as rates per 100 patient-years. *Results.* Data from 718 patients (59% female) were pooled, corresponding to over 1000 patient-years on

study. In studies where information on prior ITP treatment was collected, 100% of patients (482/483) had received ≥1 prior ITP treatment, and 38% had a splenectomy. The mean baseline platelet count from the first ITP studies that the patient enrolled in was 20×10⁹/L (SD: 16×10⁹/L). In total, 580 patients received romiplostim; 65 received placebo/SOC; and 73 received placebo/SOC in the parent study and romiplostim in the subsequent study. The median duration of exposure to romiplostim was 52 weeks (range 1-250 weeks). The average weekly romiplostim dose was 3.5 mcg/kg. Fifteen percent of romiplostim-treated patients and 22% of placebo/SOC-treated patients discontinued their parent ITP study. Adverse events were reported in 92% of romiplostim patients and 94% of placebo/SOC patients. The most frequently reported adverse events (rates per 100-patient years, romiplostim vs placebo) were: headache, (79 vs 58), contusion (60 vs 50), and epistaxis (46 vs 53). There appeared to be reductions in the rate of bleeding events in the romiplostim group compared to the placebo/SOC group, but the confidence intervals overlapped (Table 1). Adverse events of interest in the treatment of ITP with TPO mimetics include thrombosis events, bone marrow reticulin events, neoplasms, and hematopoietic malignancies. These events were analyzed in both the romiplostim and placebo/SOC groups (Table). The rate of thrombotic events was comparable between the romiplostim and placebo/SOC groups. Reticulin was detected in the bone marrow of 12 patients who received romiplostim; in 4 patients for whom post-treatment follow-up biopsies were available, reticulin grade either decreased or remained the same after romiplostim withdrawal. The rate of all neoplasms and of hematopoietic malignancies was lower in the romiplostim group than the placebo group. Two patients developed neutralizing antibodies to romiplostim but did not develop neutralizing antibodies to TPO. *Conclusions.* This integrated analysis provides long-term safety information from all available ITP romiplostim clinical studies, with some patients treated for close to 5 years. No new safety signals were identified with the long-term use of romiplostim and the adverse event profile was consistent with that previously reported.

Table 1. Adverse events.

	Placebo/SOC N=138 Pt-yr=110.0		Romiplostim N=653 Pt-yr=921.5	
	rate per 100 pt-yr (95% CI)	n	rate per 100 pt-yr (95% CI)	n
Summary of Adverse Events	1150	1271	1341	12361
Serious Adverse Events	97	107	65	599
Treatment-Related Adverse Events	153	168	139	1267
Treatment-Related Serious Adverse Events	16	18	9	78
Deaths	7	8	3	24
Adverse Events of Interest				
Bleeding Events				
Any grade	214 (187, 243)	235	193 (184, 202)	1779
≥ grade 2	48 (36, 63)	53	47 (43, 52)	433
≥ grade 3	17 (10, 27)	19	11 (9, 14)	103
Thrombosis Events	6 (2, 12)	6	8 (6, 10)	69
Bone Marrow Reticulin Events	0	-	1	12
Neoplastic Events	14 (8, 23)	15	9 (7, 11)	79
Hematopoietic Malignancies/ MDS Adverse Events	2 (0.2, 7)	2	<1 (0.2, 1)	6

Pt-yr = Total patient years on study, n = Number of adverse events

0185

CIRCULATING COLLAGEN III N-PROPEPTIDE IN PATIENTS WITH PRIMARY IMMUNE THROMBOCYTOPENIA (ITP) TREATED WITH THROMBOPOIETIN AGONISTS

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Background. Thrombopoietin receptor agonists (TpoRA) have shown high efficacy in ITP. They act by stimulating thrombopoietin receptors leading to increased platelet production. However, concerns have been raised over the increased bone marrow fibrosis (BMF) that has been reported in a number of treated patients. BMF is related to accelerated megakaryocytic proliferation thought to be due to release of cytokines, which stimulate fibroblasts leading to collagen deposition. Collagen is synthesized as procollagen precursors to be transformed into collagen after the cleavage of the N and C terminal propeptides with the procollagen peptide (PIIINP) released into the circulation. Serum PIIINP has been suggested as a marker for fibroproliferative activity in the BM in various myeloproliferative disorders. *Aims.* To assess circulating PIIINP levels in ITP patients treated with TpoRA and to explore whether PIIINP levels correlate with the grade of fibrosis in the BM. *Methods.* BM biopsies were available in 27 ITP patients treated with TpoRA. In 3 patients only pretreatment BM were available. Frozen sera were collected at different time points during treatment; samples were available in 25 patients. BM fibrosis was graded into grades 0-to-4. Serum samples were analysed for PIIINP by RIA (Orion Diagnostics, Finland). According to previous publications median PIIINP in healthy subjects was 3.4 µg/L (range 2.9-3.5). *Results.* Median age of 27 patients was 51years. Median platelet counts (Inter-quartile range IQR) at initiation of TpoRA and time of last PIIINP measurement were 16×10⁹/l (13-38) and, 59×10⁹/L (30-100), respectively. Post-treatment serum samples were available in 22 patients. Median (IQR) PIIINP was 5.6 µg/L (4.1-7.3). Median (IQR) PIIINP was 4.7 (3.7-6.0) in grad 0/1 (n=13), 5.8 (4.6-7.2) in grade 2 (n=9) and 9.1 in grade 4 (n=2). Although PIIINP seemed to increase with increasing grades of BMF, the differences were not statistically significant. PIIINP was negatively associated with the duration of treatment (Spearman's rho =0.51, P=0.016) (Figure 1). Multiple samples were available in 3 patients and illustrated the levels of PIIINP decreasing with time. Mean duration of treatment for patients with reticulin grades 0/1 and 2 were 873 days (SE138) and 452 days (SE106) respectively (P=0.02). *Summary/Conclusions.* TpoRA treated ITP patients have elevated levels of circulating PIIINP, but these did not increase significantly with increasing degrees of BMF. PIIINP levels in the TPO-RA treated ITP patients were higher than in patients with transitional myelofibrosis disorders/polycythemia vera 4.3 µg/L (1.1-8.3), but lower than idiopathic myelofibrosis 8.8 µg/L (7.4-10.7). Interestingly, we found a negative association between PIIINP and duration of treatment, In line with that, we found an inverse relation between the duration of treatment and the grade of fibrosis. These findings may indicate an initial elevation or a pre-treatment increase in fibroproliferative activity that seems to fade away with time on treatment with TPO-RA. This novel finding needs to be verified in prospective studies and its mechanism better understood in the setting of no evidence of decreased efficacy over the same time period.

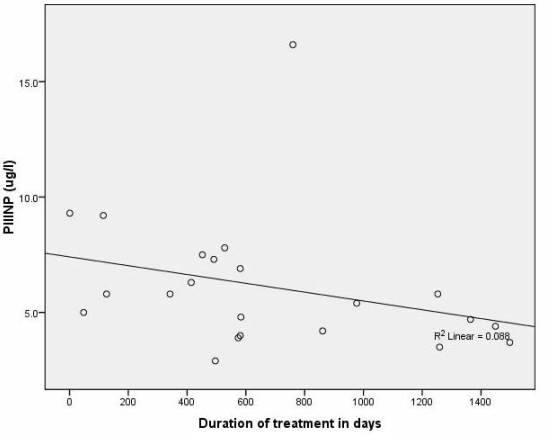

Figure 1. Association between PIIINP and treatment duration.

0186

EFFECT OF ELTROMBOPAG ON PLATELET RESPONSE, BLEEDING AND REDUCTION IN CONCOMITANT ITP MEDICATIONS IN ADULTS WITH CHRONIC IMMUNE THROMBOCYTOPENIA (ITP) AND BASELINE PLATELETS =15,000/µL

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Background. Chronic idiopathic (immune) thrombocytopenic purpura (ITP) is characterized by autoantibody-induced platelet destruction and reduced platelet production, leading to low platelet counts (<150,000/µL). Bleeding and bruising signs are largely dependent on platelet counts. Eltrombopag treatment in ITP is aimed at minimizing the risk of bleeding and bruising, and reducing or discontinuing con-

comitant ITP medications by elevating and maintaining platelets to safe levels. *Aim.* To assess effects of eltrombopag on platelet response, bleeding symptoms, and reduction in concomitant ITP medications in ITP patients with baseline platelet counts ≤15,000/μL in the 6-month placebo-controlled, phase 3 study, RAISE (Cheng et al Blood. 2008;112:400). *Methods.* Patients with chronic ITP and platelet counts <30,000/μL who failed ≥1 previous treatment received the standard of care and were randomized to placebo or 50 mg eltrombopag daily. The dose of eltrombopag was increased to 75 mg on or after day 22 for patients who did not achieve a platelet response of ≥50,000/μL. Patients achieving platelet counts >200,000/μL had their dose reduced to ≤25 mg. Bleeding was prospectively evaluated using the WHO bleeding scale. The odds of responding to eltrombopag relative to placebo were calculated using a logistic regression model for the subset of patients with a baseline platelet count of ≤15,000/μL from the RAISE study. *Results.* In RAISE, 97 patients had baseline platelet counts ≤15,000/μL (67 receiving eltrombopag and 30 placebo). Analysis of this subset showed significant clinical benefit from eltrombopag treatment, with the odds of responding significantly higher compared to placebo (P<0.0001). Response to eltrombopag 75 mg in this subset was observed in 56% of patients who required dose escalation compared to 10% of patients in the placebo group. Even though patients with a baseline platelet count >15,000/μL reported more instances of no bleeding (grade 0) compared to patients with baseline platelet counts ≤15,000/μL, the odds of bleeding were significantly reduced in the eltrombopag group with platelet counts ≤15,000/μL compared to placebo (P<0.001). Of 29 patients in the eltrombopag group with baseline platelet counts ≤15,000/μL and concomitant ITP medications at baseline, 19/29 (66%) discontinued or reduced at least 1 concomitant ITP medication compared to 4/15 (27%) in the placebo group. Of this population, 14/19 (74%) eltrombopag-treated patients did not require any rescue medication after a sustained reduction or permanent discontinuation of a baseline ITP medication, compared to 1/4 (25%) placebo-treated patient (Table). The incidence of adverse events (AE) in the overall population was similar in the eltrombopag and placebo groups with approximately 90% of patients experiencing at least 1 AE. Serious AEs in the described subset occurred in 9% of eltrombopag-treated patients compared to 30% of patients in the placebo group. *Conclusions.* The RAISE study reported increased platelet counts, decreased bleeding, and reduction in concomitant baseline ITP medications in patients with ITP treated with eltrombopag relative to placebo. This subset analysis shows that eltrombopag-treated patients with baseline platelet counts ≤15,000/μL have a less robust response but still obtain significant benefit in terms of platelet response, bleeding symptoms, and reduction in concomitant ITP medications relative to placebo.

Table. Reduction on concomitant ITP medications in patients with baseline platelet counts ≤15,000/mL.

	Placebo N=30[a] n (%)	Eltrombopag N=67 n (%)
Baseline ITP medication	15 (50)	29 (43)
Reduced/discontinued ≥1 baseline ITP medication	4/15 (27)	19/29 (66)
Permanently discontinued or sustained reduction[b]	1/4[c] (25)[d]	14/19 (74)[d]

a. One patient with missing baseline platelet count.
b. Excludes patients who subsequently required rescue medication (increase in dose of baseline concomitant medication, new ITP medication, platelet transfusion, or splenectomy).
c. One patient discontinued danazol due to serious adverse events of renal function test abnormality, alanine aminotransferase increase, hyperkalemia.
d. These values are based on the subpopulation that reduced or discontinued ≥1 baseline ITP medication.

ITP, immune thrombocytopenia.

0187

ANTIGENIC DIFFERENCES IN PATIENTS WITH TYPE 1 GAUCHER DISEASE RECEIVING VELAGLUCERASE ALFA OR IMIGLUCERASE ENZYME REPLACEMENT THERAPY IN CONTROLLED CLINICAL TRIALS

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Background. Gaucher disease results from a deficiency in the lysosomal enzyme, acid beta-glucosidase. In type 1 Gaucher disease, enzyme replacement therapy with human acid beta-glucosidase can reverse most disease symptoms. However, development of antibodies to therapeutic proteins may impact patient safety, efficacy and drug pharmacokinetics. *Aims.* To compare antibody responses in patients receiving either velaglucerase alfa or imiglucerase. *Methods.* Patients who participated in the velaglucerase alfa Phase III studies (TKT032, TKT034 and HGTGCB-039) were monitored for development of anti-drug antibodies (ADA). Of 99 patients treated, 82 received velaglucerase alfa and 17 received imiglucerase. All patients gave informed consent. Calibrated, sequential, direct bridging electrochemiluminescence (ECL) methods were developed and validated for velaglucerase alfa and imiglucerase. Samples positive in the bridging assay were confirmed and isotyped using quantitative radioimmunoprecipitation (RIP) assays (for IgG ADA) or indirect binding ECL immunoassays (for IgE, IgA and IgM). Isotype-specific goat-human hybrid controls were developed for the latter. All ADA-positive samples were tested for neutralizing antibodies (NAb) using methods based on *in vitro* inhibition of enzyme activity. All patient specimens were evaluated for ADA in parallel in a masked fashion. *Results.* ADA-positive cut-points for both therapeutics were established as 5 and 4 ng/mL in the ECL bridge and RIP assays, respectively. In study TKT032, 25 naïve patients were treated with velaglucerase alfa for 12 months and only 1 patient developed IgG ADA and NAb in response to velaglucerase alfa. In study TKT034, 40 patients who had been previously treated with imiglucerase, were treated with velaglucerase alfa for 12 months. No patient became anti-velaglucerase ADA-positive, despite the presence of 3 patients who had pre-existing anti-imiglucerase ADA at baseline. In study HGT-GCB-039, 17 naïve patients were treated with velaglucerase alfa and 17 naïve patients with imiglucerase for 9 months, with identical doses. No velaglucerase alfa-treated patient developed any ADA. Four imiglucerase-treated patients developed IgG ADA (of whom 1 developed NAb) in response to imiglucerase. *Conclusions.* Highly sensitive and equivalent methods were developed, standardized and validated to directly assess and compare patient antibody response to velaglucerase alfa and imiglucerase treatments. The results show seroconversion in 1% of patients treated with velaglucerase alfa and in 23% of patients who were treated with imiglucerase, suggesting significant antigenic differences between velaglucerase alfa and imiglucerase.

0188

ENZYME REPLACEMENT THERAPY WITH VELAGLUCERASE ALFA SIGNIFICANTLY IMPROVES KEY CLINICAL PARAMETERS IN TYPE 1 GAUCHER DISEASE: POSITIVE RESULTS FROM A RANDOMIZED, DOUBLE-BLIND, GLOBAL, PHASE III STUDY

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Background. Type 1 Gaucher disease, resulting from deficiency of glucocerebrosidase, leads to anemia, thrombocytopenia, hepatomegaly, splenomegaly and skeletal abnormalities. The current gold standard treatment is enzyme replacement therapy (ERT) using recombinant glucocerebrosidase manufactured in Chinese hamster ovary cells. Velaglucerase alfa is a new ERT being investigated for use in type 1 Gaucher disease. Unlike recombinant enzymes, it is produced by gene activation in a human cell line. *Aims.* To evaluate the efficacy and safety of velaglucerase alfa in type 1 Gaucher disease. *Methods.* Twenty-five treatment-naïve, anemic, type 1 Gaucher disease patients (age range 4-62 years) were randomized to intravenous velaglucerase alfa 60 U/kg (n=12) or 45 U/kg (n=13) every other week for 12 months. All patients gave informed consent. *Results.* At 12 months, mean hemoglobin concentration increased in both groups (60 U/kg: 23.3% increase, +2.43±0.32 g/dL, P=0.0001; 45 U/kg: 23.8% increase, +2.44±0.47 g/dL, P=0.0001), as did mean platelet count (60 U/kg: 65.9% increase, +50.9±12.×10^9/L, P=0.0016; 45 U/kg: 66.4% increase, +40.9±13.6×10^9/L; P=0.0111). Mean spleen volume decreased in both groups (60 U/kg: 50% decrease, -1.92±0.51% body weight, P=0.0032, from 14.0 multiples of normal [MN] at baseline to 5.75 MN; 45 U/kg: 40% decrease, -1.87±0.60% body weight, P=0.0085; from 14.5 to 9.5 MN) as did liver volume (60 U/kg: 17% decrease, 0.84±3.3% body weight, P=0.0282, from 1.46 to 1.22 MN; 45 U/kg: 6% decrease, -0.30±0.29% body weight, P=0.3149, from 1.40 to 1.24 MN). Velaglucerase alfa was generally well tolerated with no drug-related serious AEs, and no patient withdrew due to an AE. The most common AEs were headache, nasopharyngitis, injury, arthralgia, cough, and pyrexia. A single patient developed antibodies. *Conclusions.* In this global, multicenter study, velaglucerase alfa 60 U/kg and 45 U/kg was generally well tolerated and effective as a first-line treatment for adults and children with type

1 Gaucher disease. All clinical parameters measured demonstrated clinically meaningful improvements after 12 months, with a greater response seen with velaglucerase alfa 60 U/kg.

0189

EVALUATION OF EFFICACY AND SAFETY OF LONG-TERM ROMIPLOSTIM TREATMENT FOR PATIENTS WITH IMMUNE THROMBOCYTOPENIA (ITP) ENROLLED IN AN OPEN-LABEL EXTENSION STUDY: A PATIENT COHORT-ANALYSIS

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Background. ITP is an autoimmune disease characterized by low platelet counts due to both increased platelet destruction and suboptimal platelet production. Romiplostim, a novel peptibody that increases platelet production similarly to thrombopoietin, is approved for the treatment of adult chronic ITP and patients have been treated in an open-label extension study for more than 5 years. *Aims.* To evaluate the safety and efficacy of long-term romiplostim treatment among cohorts of patients with different disease characteristics and different lengths of exposure to romiplostim. *Methods.* Eligible patients had completed a prior romiplostim study without significant changes in their medical history. Informed consent was obtained from all patients. Romiplostim was administered once weekly by subcutaneous injection, with dose adjustments to maintain platelet counts in the target range (50 to 200×10^9/L). Patients were divided into 4 cohorts based on their date of study enrollment (corresponding to key protocol changes over time). Protocol changes over time included lowering the maximum allowed romiplostim dose and removing the baseline platelet count requirement. Exposure-adjusted adverse event rates were expressed as rates per 100 patient-weeks to account for different exposures on-study. *Results.* Overall, 291 patients received romiplostim. Certain baseline patient characteristics differed among the 4 cohorts (Table) and were most prominent in cohort 4, which contained largely nonsplenectomized patients, who enrolled immediately after completing their prior study and had higher baseline platelet counts and shorter history of ITP. Duration of romiplostim treatment in this study varied from ≤244 weeks in cohort 1 to ≤72 weeks in cohort 4. As median treatment duration increased, there was no trend for increased rates of adverse events (Table).

Table.

	Cohort 1 N = 34 6166 pt-wks	Cohort 2 N = 90 11754 pt-wks	Cohort 3 N = 31 2857 pt-wks	Cohort 4 N = 136 5113 pt-wks	Overall N = 291 25890 pt-wks
Enrollment period	April 2004 to February 2005	February 2005 to May 2006	May 2006 to October 2007	October 2007 to May 2009	-
Maximum allowed romiplostim dose at enrollment, mcg/kg	30	15	10	10	-
Study entry platelet count criteria, x10^9/L	≤ 50	≤ 50	≤ 50	none	-
Median (Q1, Q3) baseline platelet count, x10^9/L	18 (10, 24)	19 (11, 33)	13 (6, 32)	113 (55, 190)	35 (15, 96)
Splenectomy, % (n)	77% (26)	53% (48)	61% (19)	2% (2)	33% (95)
Median (Q1, Q3) duration ITP, years	9 (4, 16)	6 (3, 13)	11 (4, 15)	3 (2, 7)	5 (2, 11)
Median (min, max) duration of treatment in extension study, weeks	222 (1, 244)	151 (2, 240)	96 (3, 144)	36 (4, 72)	48 (1, 244)
Adverse event, rate (n)	27.4 (1687)	19.9 (2341)	30.5 (871)	15.1 (771)	21.9 (5670)
Serious adverse event, rate (n)	0.9 (56)	0.9 (111)	1.8 (52)	1.0 (4)	1.0 (270)
Treatment-related adverse event, rate (n)	2.3 (144)	1.4 (167)	0.9 (25)	1.3 (67)	1.6 (403)
Treatment-related serious adverse event, rate (n)	0.2 (11)	0.1 (8)	0.4 (12)	0.1 (7)	0.1 (38)
Deaths, rate (n)	0 (0)	0.1 (6)	0.1 (3)	0.1 (4)	0.1 (13)
Bleeding events, rate (n)	3.2 (200)	3.0 (353)	4.0 (113)	1.7 (89)	2.9 (755)
Platelet count ≥ 50x10^9/L, n (%)	28 (82%)	85 (94%)	27 (87%)	133 (98%)	273 (94%)

n =number of adverse events, pt-wks = total patient-weeks, rate = exposure adjusted AE incidence rate per 100 patient-weeks ((n/pt-wk)x100)

The median average weekly dose across the overall study population was 4 mcg/kg (interquartile range: 2 to 7 mcg/kg). The mean weekly dose remained stable within cohorts and was consistently highest in cohort 1, in which patients had been allowed to receive romiplostim at higher doses and had more severe disease, and was lowest in cohort 4. Almost all patients (94%) experienced a platelet count ≥50×10^9/L during the study. After the first week, overall median platelet counts remained within the target range for the duration of the study. Median weekly platelet counts among cohorts were similar to median weekly platelet counts overall and were consistently highest in cohort 4. *Conclusions.* In this study, the safety profile of romiplostim in ITP patients did not worsen with longer duration of treatment, and was similar among cohorts of ITP patients with different baseline characteristics. The ability to sustain platelet count increases with a stable romiplostim dose over months to years was not affected by changes to the protocol and patient population over time although the median dose and platelet count were slightly different.

0190

LOW DOSE RITUXIMAB AS SALVAGE THERAPY IN ADULT PATIENTS WITH PRIMARY IMMUNE THROMBOCYTOPENIA (ITP)

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Backgrounds. Rituximab (RTX) 375 mg/sqm weekly for 4 weeks has significant activity in adults with primary ITP. In this setting, several evidences support the possible use of lower doses of RTX. *Aims.* To investigate the short and long term activity and safety of low dose RTX as salvage therapy in previously treated symptomatic ITP. *Methods.* Forty-eight adult patients, median age 41 years, male/female sex ratio 18/30, median weight 74 kg (range 42-112), median platelets count 30×10^9/L, were treated prospectively with RTX at the fixed dose of 100 mg iv weekly for 4 weeks. All patients gave informed consent. Results were evaluated considering: overall and complete responses (ORR and CR: platelet level equal to/more than 50 and 100×10^9/L), the time to ORR and to CR (TTR and TCR: time necessary to reach a platelet number equal to/more than 50 and 100×10^9/L), the relapse rate, the relapse free survival (RFS: interval between initial response and the loss of the best response previously achieved) and the treatment free survival (TFS; interval between initial response and the necessity of beginning rescue therapy). *Results.* All patients completed the therapeutic program receiving the 4 infusions of RTX. ORR and CR were achieved in 29 (60%) and 19 (40%) patients, respectively. Univariate logistic regression showed that CR was associated with weight OR=0.95, CI95%[0.91;0.99] and age OR=0.96, CI95%[0.93;0.99], showing that CR probability decreases with age and weight increases. No association was found out with ORR. In the stepwise logistic regression only weight was associated with CR, OR=0.95, CI95%[0.91;0.99]. In responding patients, the median TTR and TCR were 35 days (range: 7-112) and 51 days (range: 7-150), considerably longer than those observed with standard dose in patients with similar characteristics (Haematologica 2003;88:538). The median time of observation, in responding patients, was 18 months (range 3-41 months). Sixteen out of 29 responding patients (55%), 10 out of 19 CR (53%) and 6 out of 10 PR (60%), relapsed and 14 needed further treatments. The cumulative RFS and TFS probabilities were 61% and 70% at 12 months and 45% and 51% at 24 months, respectively. Overall, therapy was well tolerated and only 2 patients experienced mild chills during the first infusion of rituximab. One patient, 1 month after the end of Rituximab, developed a transient interstitial pneumonia. No other infectious, haematological or extra-hematological complications were documented during follow up. *Conclusions.* If compared with previous results observed with standard dose RTX, in ITP, salvage therapy with low dose RTX: i) seems to lead to similar initial response rate; ii) has longer TTR and TCR; iii) yields to worse long term effect with higher incidence of relapses.

0191

SAFETY AND EFFICACY OF VELAGLUCERASE ALFA IN PATIENTS WITH GAUCHER DISEASE TYPE 1 PREVIOUSLY TREATED WITH IMIGLUCERASE: 1-YEAR, MULTICENTER, PHASE III CLINICAL TRIAL

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Background. Gaucher disease is caused by a deficiency in the enzyme glucocerebrosidase, leading to progressive accumulation of glucocerebroside within macrophages and subsequent tissue and organ damage, typically of the liver, spleen and bone marrow. Enzyme replacement therapy (ERT) reduces organomegaly and improves hematological parameters, and thus remains the preferred treatment for Gaucher disease. Imiglucerase, a recombinant enzyme produced in Chinese hamster ovary cells, has been used for treatment of Gaucher disease for some years, while velaglucerase alfa is a recently developed replacement enzyme, and is produced by gene activation in a human fibroblast cell line, with an identical amino acid sequence to the naturally occurring protein. *Aims.* To examine the safety of every other week dosing of velaglucerase alfa in patients with Gaucher disease type 1 previously receiving ERT with imiglucerase, and to evaluate changes from baseline to Month 12 in hemoglobin concentration, platelet count, and liver and spleen volume. *Methods.* In this open-label, 12-month study, patients at least 2 years of age were eligible to receive velaglucerase alfa at a dose equal to their prior imiglucerase regimen, with the infusion administered over 1 hour. Patients were eligible to receive home therapy. All patients gave informed consent. *Results.* Patients were enrolled in the US (11 sites), Europe (3 sites) and Israel (1 site). Of 41 patients enrolled, 40 received study drug (18 male, 22 female; age range, 9-71 years, 25% <18 years). Four patients had been previously splenectomized. Median prior imiglucerase use was 67 months (range 22-192 months); three patients tested positive for anti-imiglucerase antibodies prior to receiving velaglucerase alfa. Velaglucerase alfa doses were: ≤22.5 U/kg (n=14), 22.5-37.5 U/kg (n=12), 37.5-52.5 U/kg (n=7), and >52.5 U/kg (n=7). Velaglucerase alfa was generally well tolerated with most adverse events of mild or moderate severity. No patient experienced a life-threatening adverse event, 7 severe adverse events were reported in 5 patients, and 5 treatment-emergent serious adverse events were reported in 4 patients. One patient (in the 15 U/kg group) experienced an anaphylactoid reaction that led to discontinuation; no other patients discontinued due to adverse events. No patients developed IgG antibodies to velaglucerase alfa, including the 3 patients who tested positive for anti-imiglucerase antibodies at screening. Clinical parameters (hemoglobin concentration, platelet counts, and liver and spleen volume) were sustained at therapeutic levels through 1 year of velaglucerase alfa treatment (Table).

Table. Clinical parameters.

	n	Baseline median	Mean change or % change from baseline to month 12	90% CI	Clinically significant cutoffs
Hemoglobin (g/dL)	40	10.8	–0.1	–0.3, 0.1	–1, 1
Platelet count (x10^9/L)	40	162	7.0%	0.5%, 13.5%	–20%, 20%
Normalized liver volume (% of body weight)	40	1.9	0.0%	–2.6%, 2.6%	–15%, 15%
Normalized spleen volume (% of body weight)	36	0.5	–5.6%	–10.8%, –0.4%	–15%, 15%

Conclusions. Adult and pediatric patients with Gaucher disease type 1, who were previously treated with imiglucerase for a minimum of 6 months, were successfully transitioned to velaglucerase alfa, with stability in clinical measures of disease over 12 months' treatment with velaglucerase alfa.

0192

EFFECT OF INDIVIDUALIZED ELTROMBOPAG DOSING ON PLATELET RESPONSE IN PATIENTS WITH CHRONIC IMMUNE THROMBOCYTOPENIA

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Background. In the chronic idiopathic (immune) thrombocytopenic purpura (ITP) phase 2 dose-finding study, TRA100773A, eltrombopag effectively raised platelet counts in a dose-dependent manner with 70% and 81% of patients responding (platelet counts ≥50,000/μL) at 50 mg and 75 mg, respectively. The relationship between eltrombopag dose and platelet response allowed subsequent studies to individualize dose based on platelet response. *Aims.* To assess the effect of individualized eltrombopag dose modifications on platelet response in two phase 3 studies (a 6-week study [TRA100773B] and a 6-month study [RAISE]) in adult patients with chronic ITP. *Methods.* Patients with chronic ITP and platelet counts <30,000/μL who failed ≥1 previous treatment received the standard of care and were randomized to either placebo or eltrombopag at a starting dose of 50 mg daily. For patients who did not respond to eltrombopag 50 mg, the dose was increased to 75 mg on or after day 22. In the RAISE study, patients who achieved platelet counts >200,000/μL on eltrombopag 50 mg daily had their dose reduced to ≤25 mg. *Results.* In 46% (35/76) of patients in TRA100773B, the dose of eltrombopag was increased from 50 mg to 75 mg for insufficient platelet response (<50,000/μL); 31% (11/35) subsequently responded to 75 mg. In RAISE, patients who did not achieve platelet counts ≥50,000/μL on eltrombopag 50 mg had the dose increased to 75 mg. From day 29 to the end of treatment, the percentage of patients receiving the 75 mg dose ranged from 29% to 53%. In RAISE, patients who achieved platelet counts >200,000/μL on 50 mg were downtitrated to ≤25 mg. Throughout the study, approximately 20% of the patients at each visit were on a ≤25 mg dose. The percentage of patients responding relative to the doses received at each visit is shown in the Figure: (1) the proportion of patients responding on the 25 mg dose was >70% for the majority of visits because only patients who had achieved a platelet count response of >200,000/μL received the dose decrease, and (2) the proportion of patients responding on the 50 mg dose was 50% at day 22 and >70% for the majority of the remaining visits. As in TRA100773B, only patients who failed to respond to the 50 mg dose were considered for a dose increase to 75 mg; therefore, a subpopulation of harder-to-treat patients was exposed to the 75 mg dose. Despite this, response to eltrombopag 75 mg was observed after 1 week in >15% of patients and in 30% to 46% of patients throughout the remainder of the 6-month study. *Conclusions.* Individualized dosing allowed patients sensitive to eltrombopag to receive a lower dose while enabling patients who did not achieve target platelet counts to receive a higher dose. Dose increase can result in a higher number of patients achieving an increased platelet count level over a 6-month period.

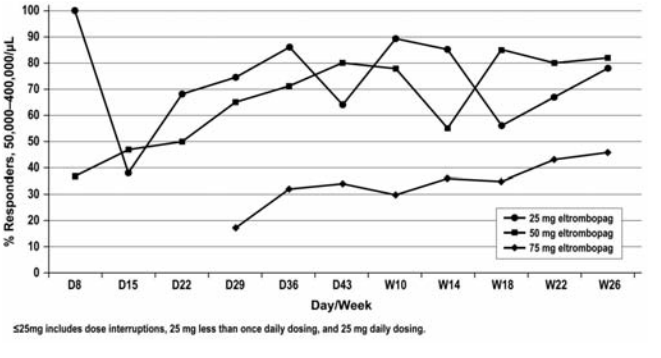

Figure. Proportion of responders by dose at each nominal isit.

0193

LOW DOSES OR HIGH DOSES OF PREDNISONE FOR CHILDREN AND ADOLESCENTS WITH UNTREATED SEVERE IDIOPATHIC THROMBOCYTOPENIC PURPURA: RESULTS OF A RANDOMIZED STUDY

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Background. Management of children with newly diagnosed idiopathic thrombocytopenic purpura (ITP) is still debatable, as well as the dosage of corticosteroids in symptomatic patients. *Aims.* This single center prospective randomized study was designed to evaluate the short, medium and long-term efficacy of treatment with two different doses of Prednisone (PDN) in children with untreated severe ITP. *Methods.* Previously untreated ITP patients aged >12 months to <20 years, with platelets (PLTs) <20,000/mmc with or without bleeding or PLTs >20,000/mmc with bleeding were stratified by age (<10 and >10 years) and randomized to receive PDN 0.25 mg/kg/day (Arm A) or PDN 2 mg/kg/day (Arm B) for 3 weeks, stopped during the fourth week. Informed consent was obtained in all cases. Treatment evaluation was performed: at the 3rd, 4th and 8th week (short time), 6th and 12th month (medium time), 5th year and in October 2009 with a follow-up questionnaire (long-term). Treatment response was defined as: complete (CR) with PLTs >150,000/mmc, partial (PR) with PLTs ≥50,000 and <150,000/mmc; no response (NR) with PLTs <50,000/mmc; relapse was defined when PLTs decreased <50,000/mmc in patients in CR or PR. *Results.* From March 1987 to February 1999, 91 patients (40 males, 51 females) with a median age of 9.6 years (range: 16 months-19.11 years) were randomized into 2 groups at our Center in Rome: 47 in Arm A and 44 in Arm B. Overall, 73 patients (80%) had PLT counts <20,000/mmc, 85 (93%) presented bleedings, 38 (42%) had a history of viral infections or vaccinations, 42 (46%) had taken drugs; 8 patients (9%) were hospitalized. As shown in Table 1, the short-term evaluation showed response in 37/44 patients (84%) belonging to Arm B compared to 12/43 patients (28%) in Arm A (P<0.01) at the 3rd week. At the 8th week the response increased to 56% in Arm A, compared to 79% in Arm B (P 0.06). Follow-up response showed no differences between Arms A and B at the 6th and the 12th month. Time to response ranged from 5 days to 18 months in Arm A and from 5 days to 5 months in Arm B (P<0.01). Five patients (Arm A) were lost to follow-up; 25/81 (31%) (Arm A: 14; Arm B: 11) relapsed after a median of 11 months (range: 2-88); 13 underwent splenectomy after 9 months to 10 years (Arm A:9; Arm B:4); 14/86 (16%) (Arm A: 8; Arm B: 6) developed an autoimmune disorder. At the 5th year, overall responders were 39/46 (85%) in Arm A and 42/43 (98%) in Arm B (P=0.01). In October 2009, 43/44 contacted patients agreed to be interviewed (median time from diagnosis: 17.6 yrs): 38/43 patients (88%) were in CR, equally distributed in Arms A and B. No long-term side effects were observed. *Conclusions.* In our study high dose corticosteroids induce a rapid PLT count increase and long-term response in ITP children. Otherwise, the different PDN dosages does not seem not to affect the medium response time, relapse rate, subsequent splenectomy and development of autoimmune disorders.

Table 1.

Evaluation Time	N° of Responders (CR + PR)/ Evaluable patients (%)		N° of NR (%)		p value
	Arm A	Arm B	Arm A	Arm B	
3rd week	12 (7+5)/ 43 (28%)	37 (25+12)/ 44 (84%)	31/43 (72%)	7/44 (16%)	<0.01
4th week	16 (7+9)/ 43 (37%)	35 (21+14)/ 43 (81%)	27/43 (63%)	8/43 (19%)	< 0.01
8th week	24 (12+12)/ 43 (56%)	33 (21+12)/ 42 (79%)	19/43 (44%)	9/42 (18%)	0.06
6th month	26 (19+)/ 42 (62%)	30 (22+8)/ 42 (71%)	16/42 (38%)	12/42 (29%)	N.S.
12th month	33 (22+11)/ 40 (83%)	34 (25+9)/ 41 (83%)	7/40 (17%)	7/41 (17%)	N.S.
5th year	39/46 (85%)	42/43 (98%)	7/46 (15%)	1/43 (2%)	0.01

0194

THE ROLE OF RETICULATED PLATELETS IN THE ASSESSMENT OF THROMBOCYTOPENIA AETIOLOGY

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Background. Reticulated platelets (RP) are platelets recently released from the bone marrow, containing more cytoplasmic RNA components than mature platelets. RP reflect the bone marrow thrombopoietic activity and is a useful new hematology parameter to distinguish thrombocytopenias caused by enhanced destruction of platelets from the hypoplasic thrombocytopenias. *Aims.* We present the RP evaluation in peripheral blood samples from patients with destructive thrombocytopenias. *Methods.* Thirty patients with thrombocytopenia: 6 Disseminated Intravascular Coagulation (DIC) (4 doing treatment); 18 Idiopathic Thrombocytopenic Purpura (ITP) (12 doing treatment); 6 thrombocytopenia of unknown cause (2 doing treatment). Control group: 15 healthy subjects. Platelet counts were performed on CELL-DYN ® Sapphire haematology analyzer using optical and immunologic (CD61) methodology. Reticulated platelet counts were done after staining with CD4K 530. SPSS 15.0 was used for statistical analysis. *Results.* Thrombocytopenic patients had a mean platelet volume (MPV) higher than control group (11 fL ±3.2 and 8.7 fL±1,2, respectively). A negative correlation (r = - 0.632) was observed between Optical Platelet (PLTo) and MPV. RP number was higher in the patient group than in the control group (8.3×10^9/L ±4.2 and 6.6×10^9/L ±1,9 respectively) as well as the RP percentage: 22.9% (minimum 1.7; maximum 98.6) vs the control group: 2.8% (minimum 1.3; maximum 4.7). Student's dependent samples t-test for the RP of the untreated patients group vs control group counts was: means difference=33.761; t=3.161 e P=0.0091. A negative correlation (r=-0.643) was found between PLTo and RPs counts. The untreated patients (n=12), irrespective of the pathology, had much higher RPs than patients already being treated (n=18) with appropriate therapeutics for each disease (mean 48.8%; minimum 6.5; maximum 98.6; and mean 6.9%; minimum 1.7; maximum 26.1, respectively). The correlation between CD61 and PLTo counts was very good (r=0.996). *Conclusions.* Patients with destructive thrombocytopenia, when compared with normal controls, have differences in the platelet size and in RP counts, and the ranges depend on the thrombocytopenia severity. Thrombocytopenic patients had increased MPV, as well as RPs, which can be explained by the fact that bone marrow, trying to compensate a low platelet number, releases more immature platelets with a higher MPV. PLTo and CD61 counts have a very strong correlation, showing that monoclonal CD61 marker is not necessary in these cases, bringing an economic benefit. A significant statistical difference was observed between non treated patients group RPs and control group. The parameter RP can be a valuable diagnostic tool in distinguishing thrombocytopenias resulting of enhanced destruction from hypoplasic thrombocytopenias, due to its simplicity, ready availability, leading to less invasive procedures with economical benefits. However, further complimentary studies should be performed.

0195

MANAGEMENT OF RELAPSED ADULT IMMUNE THROMBOCYTOPENIC PURPURA: LONG-TERM RESULTS

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Background. Adult immune thrombocytopenic purpura (ITP) is mostly a chronic disease and a considerable portion of patients experience recurrent relapses. Second-line treatment for these patients will require various treatment modalities including splenectomy. *Aims-Methods.* A total of 258 cases of patients with ITP who have been treated in our department over the past decade were reviewed retrospectively for response to second-line treatment modalities. *Results.* Of 258 cases referred, 186 newly diagnosed patients, with a median age of 51 years, required first-line therapy. Corticosteroids were used in 161 of them (87%) resulting in a complete (CR) and partial response (PR) rate of 65% and 19%, respectively. Mean and median duration of response were 43 and 16 months, respectively. Of the 186 initially treated

patients, 114 received second-line treatment. Forty eight patients were re-treated with corticosteroids and response rates were CR: 54% and PR: 23%, with a mean duration of second remission of 18 months and a median of 12 months. Corticosteroids as third (or higher) line of treatment were used in 40 patients with CR: 45% and PR: 20%; mean and median duration of response were 21 and 12 months, respectively. Response rates to corticosteroids did not exhibit significant differences between lines of treatment. A decreasing tendency in response duration was observed, but without statistical importance (Pearson correlation -0.034, P=0.8). Thirty-nine patients underwent splenectomy with a high response rate, 92% (36/39). Thirteen of these (36%) relapsed at a median of 4 (1-160) months. The 5-year response probability was 64% with a median duration of response 120 months (95% CI: 60-180). Five patients who relapsed after splenectomy responded to low-dose steroids. Rituximab was administered in 32 patients as a second or higher line of treatment in a dose schedule of 375 mg/m^2 weekly for 4 weeks. A 60% CR rate (19/32) was noted, with a mean and median response duration of 31 and 39 months, respectively (95% CI: 24-55). Ten patients (31%) maintained long term (2 to 5 years) responses to Rituximab. Re-administration of the drug in 3 cases resulted in new responses, of similar duration. No long-term side effects were observed. Vinca alkaloids (vicristine/vinblastine) were used in 46 patients. Half of them, showed some degree of short response (median 5 months). Ten patients received danazol, with a response rate of 58% which was sustained on treatment only. A small number of relapsed patients received other forms of treatment: anti-Rhesus globulin (3pts.), cyclosporine (3pts.), cyclophosphamide (5pts.) and thrombopoetin analogues (2pts). *Summary/Conclusions.* Among the usual second-line treatments for ITP, splenectomy still shows the highest response rates and longest response duration. Steroids are effective in relapsed ITP but the response is short. Rituximab can induce long-term responses in a subset of patients, without significant toxicity. Thrombopoetin analogues still require longer observation periods.

0196

HROMBOCYTOPENIA AND PLATELET INDICES IN SEPTIC NEONATES

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Background. Severe thrombocytopenia (PLT 0-30k/μl) is a common finding in septic neonates with the severity depending on the type of sepsis. Platelet indices (platelet distribution width-PDW, mean platelet volume-MPV, plateletcrit-PCT) may contribute to diagnosis and follow-up of sepsis. *Aims.* To evaluate the incidence of thrombocytopenia and the changes of the platelet indices in septic neonates in relation to the causative microorganism [gram-positive (gram+) and gram-negative (gram-)] and the gestational age. *Methods.* 154 neonates with sepsis (94 gram+, 60 gram-) were studied. Platelet count and indices were recorded on the day of positive (time 1) and the day of negative blood culture (time 2) as well as at the minimum platelet count (nadir of thrombocytopenia)(time NAD), and they were correlated with the GA and the type of sepsis (gram+ and gram-). *Results.* 99/154 neonates (46/94 gram+, 53/60 gram-) developed thrombocytopenia, that was severe in 39/99 (31/39 with Gram- sepsis). On time 1, 13 neonates had already severe thrombocytopenia (10/13 with gram- sepsis). Severe thrombocytopenia was associated with unfavorable outcome in both gram+ and gram - sepsis (P=0.02). The NAD was 2,85 days (SD 2,27) in gram+ sepsis and 3,076 days (SD 2,86) in gram- sepsis. In all GA groups, PDW levels were significantly lower in gram- sepsis (mean 17,77%; SD 1,47) than in gram+ sepsis (mean 18,74%; SD 1,91) (P=0.05). Also, MPV was lower in gram- sepsis (mean 9,45fl; SD 1,39) than in gram+ sepsis (mean 11,03fl; SD 2,72). Significantly lower values were observed in neonates with GA 32-36 weeks (P=0.02). PCT was significantly lower in gram-sepsis. *Conclusions.* Severe thrombocytopenia is more common in gram-sepsis, especially in neonates with birth weight <1000 g. It may persist despite the return of blood cultures to negative and it is associated with unfavorable outcome. In gram- sepsis, platelets remain small with normal to low levels of PDW and MPV, whereas in gram+ sepsis they tend to be large as PDW and MPV levels increase. Thus, neonates suffering gram+ sepsis possibly are able to manage thrombocytopenia better than neonates with gram- sepsis who are at high risk of developing severe hemorrhagic manifestations.

0197

INCIDENCE AND EPIDEMIOLOGYC CHARACTERISTICS OF ITP (IMMUNE THROMBOCYTOPENIA) IN ASTURIAS (SPAIN)

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ITP is considered the most frequent cause of isolated thombopenia. Very few studies are found in the bibliography about the incidence of this illness and none are found in our country. Those which are at hand not always use the same criteria and nomenclature. Recently the EHA thrombopenia group has published an agreement for the standardization of the terminology in this patients. Given all that and in accordance with the nomenclature suggested by the EHA thrombopenia group, we have planned the research of the patients with isolated thrombopenia who come to our department. *Objectives.* to determine the incidence of isolated thrombopenia and ITP in the asturian population in the IV sanitary area. To analyse which diagnosis have derived from the research on this patients. To describe the socio-demographic and clinical characteristics of patients with isolated thrombopenia and ITP. *Methods.* descriptive and retrospective study of the patients who came for the first time to be treated as an out-patient for isolated thrombopenia for a period of three years excluding those under 18 years old. The data were collected in an Excel data base and were analysed with an statistical package SPSS. *Results.* Incidence of isolated thrombopenia: 138.6/million people/year. Incidence of ITP: 66.8/million people/year. Number of cases of isolated trombopenia: 138. Mean age: 56.8 years old. 73 males/ 65 females. Derived diagnosis: primary ITP: 48, ITP linked to autoinmune disease: 15, ITP induced by drugs: 3, hepathopaty: 19, infection: 8, hemathological illness: 7, transitory thrombopenia non subsidiary: 5, macrothrombocitopenia: 2, bone marrow toxicity: 2, splenic abduction: 1, multifactorial: 1, in process of diagnostic: 6. Total cases of ITP: 66. Mean age: 54.1 years old. Distribution by sex according to age: >60 years 17 males/10 females; <60 years 14 males/ 25 females (P=0.02). Casual diagnosis in 28.78% of the cases. The presence of haemorrhage at the moment of the diagnosis was meaningfully bigger in patients with 20×10^9 platelets/L than in those of bigger numbers (purple 94.7%, others 21.1%). Treatment: corticoids 34.8%, gammaglobulins: 7.9%, splenectomy: 3%, transfusions 4.5% and others 12.1%. In the last visit 39.9% were found in complete response, 57.57% in response and 3.03% corticoid-dependents. Comparing ITP with other thrombopenias: they present more haemorrhage (purple), fewer number of platelets at the time of the diagnosis and bigger percentage of antiplatelet antibodies. Primary ITP with respect to the autoinmune associated one, presents more purple and fewer numbers of of platelets at diagnosis. *Conclusions.* The incidence of isolated thrombopenia in our study is 138.6/million people/year and of ITP is 66.8/million people/year, ligthly superior to the published. In our series ITP is the main cause of isolated thrombopenia; it is more frequent among women in young population and among men in population older than 60 years old. 28.8% are accidental diagnosis. With respect to other thrombopenias ITP has fewer platelet counts at the time of the diagnosis, more frequency of purple and bigger percentage of antiplatelet antibodies.

0198

NATURAL COURSE OF CHILDHOOD CHRONIC IDIOPATHIC THROMBOCYTOPENIC PURPURA

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Background. Acute idiopathic thrombocytopenic purpura (ITP) is common disorder in childhood with favorable prognosis. But about 20% of ITP children have persistent thrombocytopenia under 150,000/μL over 6 months (chronic ITP). There is no drug for cure of chronic ITP so far despite of various remedies including steroid, immunoglobulin (Ig), anti-D Ig, and rituximab. *Aims.* We analyzed the natural course of childhood chronic ITP after long term follow up in order to predict the prognosis of them. *Methods*. We reviewed the medical records of chronic ITP children from 1988 to 2009 in Kyungpook National University Hospital. We excluded the patients who had secondary causes of thrombocytopenia like lupus, hepatitis, aplastic anemia, hematologic malignancy, or von Willebrand disease. *Results.* There were total 244 ITP children and 59 among them (24.2%) were chronic (male: female = 30:29) in spite of

being treated with steroid, Ig, or anti-D Ig. Two underwent splenectomy, so 57 children were involved in this study. The initial platelet count was mean 23,000/μL (3,000/μL~91,000/μL) and their age at diagnosis was 5.4 (0.4~14.7) years. The mean follow up period was 5.2 (0.7~16.8) years. We classified them into 3 groups (NP, normal platelet count group; LP, low number of platelet but over 50,000/μL group; VLP, very low platelet level under 50,000/μL group) according to their last blood tests after long term monitoring. Finally, 21 of 57 chronic ITP children (36.8%) were affiliated to NP, and their last platelet count was 207,000/μL (150,000/μL~317,000/μL). In process of time, NP was increased (10.3% after 2 years, 27.6% after 4 years, and 36.2% after 8 years of observation). The mean timescale for recovering was 3.5 (0.7 ~ 8.3) years and the mean age at cure was 8.6 (1.2~18.6) years. And they were tracked for mean 5.2 (0.9~12.2) years. Other 20 children (35.1%) were belonged to LP and their last number of platelet was 81,000/μL (50,000/μL~128,000/μL). The other sixteen children (28.1%) were VLP. Their mean platelet level at diagnosis (15,000/μL, ranging from 4,000/μL to 46,000/μL) was lower in comparison with those of NP (25,000/μL) and LP (27,000/μL). And their mean tracing period (3.9 years, ranging from 0.8 to 15.8 years) was shorter than NP (5.2 years) and LP (6.4 years). So it is necessary to observe VLP closely for some years ahead. Their last platelet count was 24,000/μL (9,000~45,000/μL). *Conclusions*. Among chronic ITP children, 36.8% were recovered after long term follow up and total 71.9% showed the platelet count over 50,000/μL without any problems in everyday life. Therefore it is important to examine them regularly over several years, and perform splenectomy selectively.

0199

HIGH DOSE DEXAMETHASONE AS FRONT LINE THERAPY FOR ADULT PATIENTS WITH PRIMARY IMMUNE THROMBOCYTOPENIA

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Background. Prednisone (PDN) 0.5 to 2 mg/kg/d is generally considered the first standard line therapy for adult patients with previously untreated symptomatic Primary Immune Thrombocytopenia (ITP). The therapeutic outcome with PDN is dismal since, despite initial high response rate, most of patients lose their response after PDN withdrawn and suspension, necessitating rescue therapy. In this setting, previous phase II studies highlighted the possible higher therapeutic activity of one or more courses of high dose dexamethasone (HD-DXM; 40 mg/d for 4 days) to achieve and maintain hematological sustained remission. In particular, a recent report (Blood 2007;109:1401-7) indicated that three or four bi-weekly HD-DXM courses may provide 85% initial response rate and 60% relapse-free survival at 15 months in adult patients (≥18 years). *Aims.* To evaluate the activity and safety of HD-DXM in newly diagnosed adult patients with ITP. *Methods.* Twenty patients, median age 44 years (range 19-74), with previously untreated symptomatic ITP were treated prospectively with oral HD-DXM 40 mg/d for 4 consecutive days to be repeated every 15 days for three total courses. Patients median baseline platelet count was 10×10^9/L. Response assessment was evaluated considering the rate of initial overall and complete response (OR, CR; platelet level ≥ 50×10^9/L and ≥100×10^9/L after the end of the third HD-DXM course) and the relapse rate. *Results.* Fifteen patients completed the therapeutic program as initially scheduled while 5 had an early interruption because of insufficient response and need of other therapy. Ten, 4 and 2 patients necessitated further steroids or IVIG administration during the interval between the three HD-DXM courses. The rate of initial OR and CR was 40% each (8/20). The median period of observation in responding patients was 8.5 months (range 1-25). Two out of 8 responder patients relapsed; therefore 6 out of the initial 20 patients (30%) achieved and maintained response. One patient developed pneumonia during HD-DXM while no other relevant side effects were documented. *Conclusions.* In this single institution experience, treatment with HD-DXM did not seem to significantly improve ITP outcome if compared to the results expected with standard steroid therapy .

0200

PREDICTION OF COURSE OF ITP IN CHILDREN

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Immune thrombocytopenic purpura (ITP) is the most common acquired bleeding disorder of childhood. The prognosis of patient's ITP course at time of diagnosis will allow to individualize the therapy. *Material and Methods.* We examined 264 children at age 1-18 years with *de novo* ITP treated at BRCPOH. All patients received written information about the study and gave informed consent. Level of circulating $CD3^+$, $CD3^+HLA^-DR^+$, $CD4^+CD8^-$, $CD4^-CD8^+$, $CD19^+$, $CD16^+CD56^+$, $CD3^+CD16^+CD56^+$ lymphocytes were measured at diagnosis in all patients by flow cytometric analysis. Serum concentration of IgG, IgM, IgA was determined by nephelometric method. Serum concentration of total IgE was determined by chemiluminescence's technique with the use of test-system of anti-IgE-monoclonal antibodies. To build prediction rules within subgroups decision tree technique was applied. *Results.* There were 126 boys and 138 girls in the survey. There were 147 children (55,6%) with the acute form of disease (group I) and 117 children (44,4%) succeedingly developed chronic form of disease (group II). Median of age of children of group I is 3,5 years, group II - 7,5 years. Relative risk of developing chronic ITP in subgroups with age ≥6 years is 2,05 (1,23÷3,44) (F-test (two-tailed), P<0.01). Absolute and relative number of B-lymphocytes is higher at children of group I in comparison to the children of group II (P<0.01). We did not find significant differences in the presence of IgG, IgM, IgA at children of the analyzed groups. Another important fact is the significant increase of concentration of IgE at children of group II in comparison to group I (P=0.000006). *Conclusion.* We developed the decision-making model of prediction of the course of *de novo* ITP at children: Acute ITP: 1) at boys older than 6 years old with initial total serum IgE less than 70 ME/l; 2) at girls older than 6 years with initial total serum IgE than 70ME/L and IgG less than 12 g\L; 3) at children of 1-6 years old with initial total serum IgE less than 35ME/l and $CD19^+$cells more than 12%; 4) at girls of 1-6 years old with initial total serum IgE less than 35ME/L and $CD19^+$cells less than 12%; 5) at children with initial total serum IgE less than 60ME/l and $CD19^+$cells more than 12% and platelet count on Day 28 less than 150×10^9/L; 6) at children with initial total serum IgE less than 60 ME/l and platelet count on day 28 more than 150×10^9/L. Chronic ITP: 1) at children older than 6 years old with initial total serum IgE more than 70 ME/l; 2) at children of 1-6 years old with initial total serum IgE more than 35 ME/L ; 3) at girls older than 6 years old with initial total serum IgE less than 70 ME/L and IgG more than 12 g/L; 4) at boys of 1-6 years old with initial total serum IgE less than 35ME/l and CD19+ cells less than 12%; 5) at children with initial total serum IgE less than 60 ME/l, $CD19^+$cells less than12% and platelet count on day 28 less than 150×10^9/L.

0201

EFFICACY AND SAFETY OF SPLENECTOMY IN IMMUNE THROMBOCYTOPENIC PURPURA: RESULTS OF 38 CASES

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Background. Glucocorticoïds are the first-choice treatment of immune thrombocytopenic purpura (ITP), resulting in a good response in 70-80% cases. However, relapses occur in the third of cases. For these and for selected patients with acute refractory ITP, splenectomy may produce a good response. We report the outcome of ITP splenectomized patients. *Patients and Methods.* We retrospectively analysed the data of 38 patients (children and adults) who underwent splenectomy for ITP between 1995 and 2008. For these patients we studied the indication of splenectomy, the time between diagnosis and splenectomy, response to splenectomy and the possible complications related to splenectomy (thrombosis, infection[3DOTS]). *Results.* Between 1995 and 2008 thirty eight patients (11 children and 27 adults) were splenectomized for ITP. The mean time between diagnosis and splenectomy was 24 month. 35 patients had a chronic cortico-resistant or cortico-dependent ITP. Three patients with cortico-resistant ITP were splenectomized two months after the diagnosis: pregnancy (one case) and serious hemorrhagic events (two cases). The overall response rate was 86% (24 adults and 9 children). Two women (8%) and two children (22%) relapsed. They were retreated with Glucocorticoïds and achieved a good persistent response. One patient developed a thrombosis after splenectomy.

We have noted any serious infection during follow-up. *Conclusions.* This study shows that splenectomy is a safe and an effective procedure for chronic ITP with a high rate of good response as described on the litteraure (60-80%). However a long follow-up was needed to detect tardive relapse. Splenectomy is and still the 2nd line choice treatement in our country and further studies were required to establish whether other treatment of chronic ITP, such as anti-D, anti-CD20 and analogue of thrombpoïetine will delay splenectomy.

0202

RITUXIMAB IN IMMUNE THROMBOCYTOPENIC PURPURA: TRANSIENT RESPONSES, LOW RATE OF SUSTAINED REMISSIONS AND POOR OUTCOME OF REFRACTORY PATIENTS

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Management of patients with immune thrombocytopenia (ITP) refractory to standard treatment is difficult. Recent studies show that rituximab, a chimeric anti-CD20 monoclonal antibody, is useful in the treatment of ITP. *Methods.* We retrospectively studied 24 patients (16 females) who received 29 rituximab treatments (off label) for relapsed or refractory ITP. *Results.* Nineteen patients had primary (idiopathic) disease and five patients had secondary ITP (3 CLL, 1 NHL, 1 SLE). Patients had received a median of 3 treatment regimens before (range 1-8) and 11 patients had prior splenectomy. Responses were achieved in 19 of 29 (66%) treatments. The median time to response was 3 weeks (range 1-20) from the start of therapy and median duration of response was 13 weeks (range 1 week -55 months). Responses were mostly short lived and after a median follow-up of 22 months (range 2-70), 10 (34%) responses were sustained after 6 months, 7 (24%) responses sustained after one year and only 5 patients continued to have a response at the last visit after 8, 10, 24, 30 and 54 months of follow-up. Previous splenectomy correlated with a poor response (p 0.034). Patients who failed rituximab and had prior multiple treatments including splenectomy, had a poor outcome of further therapies. *Summary.* We conclude that rituximab is well tolerated and is useful in some patients with relapsed or refractory ITP; however, only about one fifth of patients achieved sustained remissions. Patients refractory to rituximab have a poor response to further treatment.

0203

LOW-DOSE RITUXIMAB IN ADULT PATIENTS WITH PERSISTENT/CHRONIC PRIMARY IMMUNE THROMBOCYTOPENIA: A SINGLE CENTER EXPERIENCE

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Background. It is known that treatment with standard weekly dose of Rituximab (375 mg/m^2) for 4 weeks is effective in primary immune thrombocytopenia (pITP); recent literature data support the use of lower doses of Rituximab in these patients. *Aim.* To investigate the efficacy of low-dose Rituximab in patients with persistent/chronic pITP. *Patients and Methods.* Ten adult pITP patients (5 males, 5 females; median age 40.5 years [19.8-63.8]) were treated with Rituximab (weekly dose 100 mg i.v., for four consecutive weeks). Median time between diagnosis and start of Rituximab was 1.6 years (0.5-12.4). All patients had already received at least one line of therapy (median 2, 1-3): standard-dose prednisone, pulsed high-dose dexamethasone, immunoglobulins, splenectomy. At the start of Rituximab, the median platelet count was 17×10^9/L (5-30×10^9/L). Response definition was as follows: complete response (CR), platelet count ≥100×10^9/L; partial response (PR) >50 <100×10^9/L; minimal response (MR) >30 ≤50×10^9/L; no response (NR) ≤30×10^9/L. After completing therapy, patients were evaluated for platelet count after 1 and 3 months, and thereafter every 3 months. Peripheral blood B lymphocytes were evaluated by flow-cytometry as CD20$^+$ cells before treatment, 1 and 3 months after stopping therapy, and then every 3 months up to recovery. *Results.* One month after the end of Rituximab, all patients were evaluable for response: seven responses (4 CR, 2 PR, 1 MR, 70%) and 3 NR (30%) were observed. At the 3rd month's evaluation, responses were 6 (4 CR, 2 PR 60%), NR were 2 (20%) and relapses were 2 (20%). Median follow-up of all treated patients was 5.5 months (3.3-19.7); median follow-up of all responder patients was 5.5 months (3.3-16.3). At the last control, 6 patients were in persistent responses (4 CR, 2 PR 60%), while 4 were NR. Before starting therapy, all cases were valuable for flow-cytometry studies. The median baseline value of peripheral blood CD20$^+$ B cells was 289.5/μL (85-856). One month after completing treatment, all cases showed absence of circulating CD20$^+$ cells. At the last available control, all patients had still not recovered the baseline CD20$^+$ cells count. Only one side effect a case of influenza-like illness was observed. *Conclusions.* At the last follow-up, 6 out of 10 patients with persistent/chronic pITP (60%) showed a persistent response to low-dose Rituximab (4 CR, 2 PR). The response seems to be independent from the post-therapy CD20$^+$ cells counts. No serious infections were observed during the clinical follow-up. No patient had to stop therapy because of severe side effects.

0204

ADULT IMMUNE THROMBOCYTOPENIC PURPURA: MANAGEMENT AND LONG TERM OUTCOME OF 168 CASES

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Background. Immune thrombocytopenic purpura (ITP) is an acquired disease characterized by an immunological peripheral platelet destruction. ITP occur at any age and had a more chronic evolution in adult. We report the therapeutic results and outcome of adult ITP. *Patients and Methods.* Between January 1995 and December 2008, we retrospectively analyzed the data of 168 adults with ITP diagnosis. The first treatment was Glucocorticoïds: Prednisone® 1 mg/kg/day during 4 weeks followed by a degression, dose and duration were adapted to the elderly and the diabetics. The evaluation at days 28 are complete response (CR) if platelet count is higher than 100 G/L, partial response (PR) if platelet count is higher than 50 G/L and failure if platelet count is lower than 50 G/L. For patient with chronic evolution (up on six month) we evaluated various therapeutic lines such as high dose of glucocorticoïds (bolus of methylpredisolone), Vincristine®, Danatrol® and splenectomy. *Results.* One hundred sixty eight adults (114 women and 55 men) with a median age of 43 years (16 to 100 years) were followed over fourteen year period. One hundred forty two (84%) patients achieved good response (CR+PR) to prednisone®. Fifty one (36%) patients relapsed in a median of 11 months of response. Sixteen patients received bolus of solumedrol in 2nd and 3rd line with 43% PR rate. Five patients received Vincristine® with only one CR. Ten patients received Danatrol® with two persistent CR. Twenty five patients underwent Splenectomy in 2nd, 3rd and 4th line, the response rate was 87%. A chronic evolution was noted in 42% cases particularly in women. Finally, we have noted any death related to thrombopenia or treatement. *Conclusions.* Glucocorticoïds is the first-choice treatement for ITP resulting in a response in 84% as like as reported studies. However relapse occurs in the third of cases. Splenectomy remains the treatement of choice in 2nd line in our country comparing to the cost and results of rituximab. We have noted more frequent chronic evolution particularly in women.

0205

TREATMENT WITH RITUXIMAB IN REFRACTORY ITP PATIENTS. EXPERIENCE AND FOLLOW-UP IN 9 PATIENTS BETWEEN 2005-2009

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Background. Refractory immune thrombocytopenic purpura (ITP) is defined as persistent severe thrombocytopenia (<20.000 platelets) for more than 3 months and no response to conventional first-line therapies. Despite the fact that splenectomy is still considered the 'gold standard' treatment in many countries for the management of chronic ITP the risk of severe morbidity resulting from surgery or overwhelming post splenectomy infections and late relapse that persists during long-term follow-up represents a major concern. Rituximab could be an effective and safe splenectomy-avoiding strategy in adults with chronic ITP. Responses rates about 40% have been demonstrated with minimal side effects, suggesting it would be an effective first-line therapy in refractory ITP associated with autoantibody-mediated disorders (AD). *Aim.* To retrospectively collect data on refractory IPT patients treated with Rituximab in our Hospital to evaluate the efficacy and safety of this therapy regimen. *Patients and Methods.* 9 adult patients were treated with Rituximab (R) between 2005-2009 (7 female; 2 male). Five patients received Rituximab post splenectomy and four prior to splenectomy.

Three patients were previously diagnosed with AD (1 Sjögren syndrome, 1 systemic lupus erythematosus, and 1 polyserositis). All patients received steroids or intravenous gamma globulin (IVIG) in addition to R in order to maintain adequate platelet levels. Rituximab (375 mg/m^2; Roche France, Paris, France) was infused intravenously weekly for 4 weeks. No complications during the infusion or the follow up time were observed. *Results.* Platelet count greater than 150,000 was considered a complete response (CR), and platelet count greater than 50,000 was a partial response (PR). CR was achieved in 100% (5) of the patients who received Rituximab after splenectomy (Table 1) in 1.6 months. Relapse occurred in 40% (2) of these patients, who then received a second dose of Rituximab. It was well tolerated and they responded again. Only 50% (2) of the patients who received Rituximab before splenectomy (Table 2) achieved CR (they were treated earlier) whereas the other 50% (2) achieved PR and subsequently, 75% of these patients relapsed. One received a second dose of Rituximab and achieved PR, another was splenectomized and is in a real CR and the last one remained refractory. *Conclusions.* in our experience, Rituximab is a good treatment for refractory ITP. According to new guidelines, we also observed that it is achieved more qualitative and long-term responses when it is used after splenectomy. However, it is also an effective treatment for patients who could not be splenectomized. Due to the wide variety of responses in these patients randomized clinical trials in splenectomized and non- splenectomized patients with refractory ITP is needed in order to establish new guidelines.

Table 1. Patients who received Rituximab after splenectomy. m: months; w: weeks; R: rituximab; CR: complete response; PR: partial response.

	Time Dx → R	Response	Time R →response	Relapse	Time R→relapse	Response 2ª dose	Time R →response	Time of mantained response
1	20 m.	CR	2 m.	No	-	-	-	3 m.
2	32 m.	CR	3 m.	Yes	7 m.	CR	1 m.	34 m.
3	104 m.	CR	3 w.	Yes	9 m.	PR	2 w.	6 m.
4	168 m.	CR	1 m.	No	-	-	-	45 m.
5	204 m.	CR	1 m.	No			-	15 m.

Table 2. Patients who received Rituximab before splenectomy. *CR after splenectomy. m: months; w: weeks; R: rituximab; CR: complete response; PR: partial response.

	Time Dx → R	Response	Time R →response	Relapse	Time R→relapse	Response 2ª dose	Time R →response	Time of mantained response
1	3 m.	CR	3 m.	Yes	1m.	*	-	16 m.
2	3 m.	CR	3 m.	Yes	3 w.	-	-	Refractory
3	50 m.	PR	1 w.	No	-	-	-	6 m.
4	92 m.	PR	1 w.	Yes	6 m.	PR	1 w.	13 m.

0206

IMPACT OF CYTOKINE LEVELS ON HRQOL IN PATIENTS WITH ITP

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Background. Immune ThrombocytoPenia (ITP) is a heterogeneous acquired autoimmune disorder in which antibodies and T cell mediated effects under cytokine influence combine to create variable levels of platelet destruction. In addition to the autoimmune features and presumably explained by them, fatigue-related symptoms are commonly seen in ITP, seem to fluctuate with treatment and often disappear when platelet counts increase. *Aims.* This study investigated potential clinical effects of IL-6, IL-8 and TNF-α levels on ITP patients and correlated these findings with Health Related Quality of Life (HRQoL). *Methods.* Patients with chronic ITP older than 15 years old were enrolled in 2009 at the Platelet Disorders Center at the Weill-Cornell Medical Center, NY. They completed the Short-Form 36 questionnaire (SF-36), a well-standardized instrument and Multidimensional Fatigue Inventory (MFI), a specific fatigue-related questionnaire. Laboratory testing included CBC, immature platelet fraction (IPF), TSH, ferritin, iron + TIBC, ESR, CRP, blood IL-6, IL-8 and TNF-α levels. Non-parametric measures of correlation were performed using the Spearman' rank correlation coefficient and differences between subgroups were analyzed by Mann-Whitney-Wilcoxon test. *Results.* 61 patients with a mean age of 52 years (61% women) were enrolled. The mean platelet counts were 86,000/uL (range of 4,000-352,000/uL). 52.4% had had previous splenectomy and 65.6% were classified as refractory ITP either by failure of response to splenectomy (81%) or relapse (19%). Sixty-six% of the patients were on treatment: 65% Tpo-R agents and 35% 'Other' (5% steroids, 12.5% Rigel, 10% IVIG+solumedrol, 2.5% rituximab and 5% Danazol/Azathioprine). IL-6 serum levels above the upper limit of normal (>1.7 pg/mL) were found in 42.6% of the patients (n=26), and within this subgroup the lowest levels belonged to splenectomized patients. All patients in remission (n=4) had normal levels of IL-6. Statistically significant negative correlations were found between the age and physical function domain of SF36 (P<0.0001). For IL-6 levels, significant negative correlations were found on 5 domains of SF-36 adjusted by BMI, age and gender; and positively for one scale of MFI (see table). ESR was negatively correlated with SF-36 in the same domains as IL-6 (the highest was with physical function; P=0.0097). CRP was only negatively correlated with role emotional domain (P=0.0114). MFI had a positive correlation with ESR on physical fatigue scale (P=0.0011) and reduced activity (P=0.0079) but was not correlated with CRP. Splenectomy status, gender, platelet counts, MPV, IPF absolute, IPF%, ferritin, TSH, IL-8 and INF-alpha were not correlated with other scores. *Summary/Conclusions.* The present study demonstrated that a substantial subset of ITP patients has high levels of IL-6 and these levels were correlated with worse performance on HRQoL measurements. ESR was also correlated with certain scales suggesting that besides thrombocytopenia, ITP may be characterized as a sub-clinical inflammatory state which explain its constitutional symptoms. We conclude that high levels of cytokines such as IL6, derived from the reticuloendothelial system which is chronically activated in ITP, may result in a cytokine-induced behavior including fatigue observed on ITP patients.

Table 1.

Patient report outcome and IL-6 levels			
Statistical significant correlation		No statistical significant correlation	
SF-36 v1	**p value**	**SF-36 v1**	**p value**
Role Physical	0.0035	Mental summary	0.18
Role emotional	0.02	Physical summary	0.22
Vitality	0.022	Mental Health	0.38
Social Functioning	0.031	Physical Functioning	0.41
Bodily Pain	0.034	General Health	0.77
MFI	**p value**	**MFI**	**p value**
Physical fatigue	0.0096	General fatigue	0.1498
		Reduced activity	0.0561
		Reduced motivation	0.4172
		Mental fatigue	0.7332

Cytogenetics and molecular diagnostics

0207

THE PROGNOSTIC VALUE OF MONOSOMAL KARYOTYPE IN THE CONTEXT OF RECURRENT GENETIC ABNORMALITIES: AN ANALYSIS OF 2,832 ADULT PATIENTS WITH NEWLY DIAGNOSED AML ENROLLED ON FIVE AMLSG TREATMENT TRIALS

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Background. Recently, the cytogenetic category "monosomal karyotype"(MK) has been proposed in acute myeloid leukemia (AML) defined by the presence of one single monosomy (excluding isolated loss of X or Y) in association with at least one additional monosomy or structural chromosome abnormality (excluding core binding factor [CBF] AML) (Breems et al. 2008). MK was associated with a dismal outcome. *Aims.* To determine the prognostic impact of MK, in relation to the current World Health Organization (WHO) AML classification. *Methods.* The study included 3,174 adult AML patients (median age, 54.5 years; range, 16-85 years) entered on 5 AMLSG protocols between 1993 and 2008. In 2,832 of the 3,174 (89%) patients cytogenetic analysis could successfully be performed. Data on the mutation status of NPM1 and FLT3 (internal tandem duplication [ITD] and tyrosine kinase domain mutation [TKD]) was available in 2,832 (89%) and 2,604 of 3,174 (82%) patients, respectively. 1,478 of the 2,832 (52%) patients had an abnormal karyotype; after exclusion of isolated losses of sex chromosomes (n=23), CBF AML (n=311) as well as t(15;17) (n=109), 1,035 cytogenetically abnormal cases were considered for further analysis. Median follow-up for survival was 3.7 years. *Results.* 294 (28%) cases exhibited a MK and 348 a complex karyotype defined by ≥3 abnormalities (CK; 34%), with 229 cases categorized as having MK and CK. 138 (47%) MK cases had one autosomal monosomy (AM), 156 (53%) had two or more AM. MK cases harbored the following recurrent genetic abnormalities: inv(3)/t(3;3), n=34 (12%); t(6;9), n=2 (1%); t(v;11q23), n=7 (2%); mutated NPM1, n=4 (1%). In 13 (4%) MK cases, an unbalanced translocation with formation of a derivative chromosome led to a formal monosomy in the ISCN karyotype designation, but in fact without loosing the whole chromosome. In MK patients, complete remission rate was 34%, and 3-year overall survival was 9% (95%-CI, 6-14%). Among patients with CK, the additional presence of a MK was associated with an inferior prognosis (P<0.0001), whereas among patients with MK, the presence of a CK only had a marginal impact on outcome (P=0.03). With respect to the number of AM within the MK category, patients with two or more AM had an inferior outcome compared with one AM (P=0.01). We were interested whether MK retained its prognostic significance when cases with recurrent genetic abnormalities (WHO 2008) and those with derivative chromosomes containing part of lost chromosomes were excluded (MK-R). Again, MK-R conferred a dismal prognosis in patients with CK (P=0.001). In multivariable Cox regression analyses in patients with at least one cytogenetic abnormality (n=1,035), MK-R retained its impact as an independent adverse prognostic factor (HR, 2.0; 95%-CI 1.54-2.60). *Conclusions.* In this large cohort of adult AML patients, MK was an independent adverse prognostic factor, even when cases with recurrent genetic abnormalities or derivative chromosomes were excluded.

0208

PROGNOSTIC IMPACT OF MINIMAL RESIDUAL DISEASE (MRD) MONITORING DURING THERAPY AND FOLLOW UP IN NPM1 MUTATED ACUTE MYELOID LEUKEMIA: RESULTS OF THE AML STUDY GROUP (AMLSG)

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Background. Mutations in the *nucleophosmin 1* (*NPM1*) gene represent the most frequent gene mutations in acute myeloid leukemia (AML), with highest incidence (50-60%) in cytogenetically normal (CN)-AML. So far, there are no studies evaluating the prognostic value of *NPM1* mutation (*NPM1mut*) based assessment of minimal residual disease (MRD) in a larger cohort of younger AML patients (pts) enrolled on prospective clinical trials. *Aims.* To evaluate the prognostic value of *NPM1mut* MRD levels in younger (16 to 60 years) AML pts harbouring NPM1 mutations type A, B or D. *Methods. NPM1mut* specific, RNA based real-time PCR was applied to assess MRD in a total of 1100 samples [bone marrow (BM), n=1083; peripheral blood, n=17] from 214 AML pts who were enrolled in the AMLSG 07-04 and AML HD98A treatment trials of the AML Study Group. Dilution series showed a maximum sensitivity of 10-6 and high specificity as no wildtype NPM1 could be detected. *Results.* Pretreatment transcript levels were not associated with clinical characteristics (e.g., age, white cell counts, BM blasts) and did not impact relapse-free (RFS) and overall survival (OS). For evaluation of the prognostic impact of MRD levels during therapy we performed multivariate analysis at several time points during and after treatment. *NPM1mut* levels as continuous variable were significantly associated with RFS and OS after induction I (P=.0024; P=.008), induction II (P<.001; P<.001), consolidation I (P<.001; P<.001) and at the end of treatment (P<.001; P<.001). For the two clinically important MRD checkpoints, namely after double induction and at the end of therapy, achievement of PCR negativity highly discriminated two prognostic groups (P<0.001). In our study molecular relapse during follow up was defined by a threshold value of 500 *NPM1mut*/10^4 abl since some patients in continuous complete remission showed intermittent low *NPM1mut* expression in BM. All 26 pts exceeding this threshold relapsed after a median interval of 61 days (range, 11 to 709 days). However, in 5 pts (10% of all evaluated pts) relapse prediction failed due to inadequate increase of *NPM1mut* expression levels (n=2) or complete loss of the NPM1 mutation at the time of relapse (n=3). *Conclusions. NPM1mut* expression levels during therapy, in particular at early time points (after induction I and II) are of prognostic importance in *NPM1mut* AML. During follow up, a threshold value was defined that allows the early prediction of clinical relapse and may guide preemptive treatment in future clinical trials.

0209

GATA-2 OVEREXPRESSION IS A NOVEL POOR PROGNOSIS MARKER IN ACUTE MYELOID LEUKEMIA PATIENTS

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Acute myeloid leukemia (AML) is a heterogeneous group of hematological neoplasias associated with accumulation of acquired genetic alterations and epigenetic changes in hematopoietic progenitor cells. The GATA-2 (3q21) gene codes for a transcription factor with an important role in the hematopoiesis. GATA-2 is widely expressed in progenitor cells and its level gradually declines during cell differentiation. Moreover, GATA-2 is essential for the production and expansion of hematopoietic stem cells, and for their proliferation and survival in adult

bone marrow. There is evidence that GATA-2 deregulation has a role in leukemia, although so far there are few data. Our aim was to investigate the prevalence and prognostic impact of GATA-2 overexpression in AML. We analyze the expression of GATA-2 by real-time quantitative PCR in a series of 259 patients with AML at diagnosis, and evaluated its association with well-known clinical, cytogenetic and molecular prognostic factors. GATA-2 was overexpressed in 97 out of 259 patients (37.5%), confirming that it is a recurrent event in AML; moreover, it was associated with normal karyotype (NK) (45/105, 43%) and 3q aberrations (11/20, 55%). These results prompted us to further study the molecular characteristics of patients with NK. GATA-2 overexpression was significantly associated with FLT3-ITD (58.8% vs. 32.8%; P=0.017) and NPM1 mutations (54% vs. 23.5%; P=0.005). Significant differences were also found when patients were divided into 4 groups according to the mutated phenotype of these genes: both wild type (17.4%), FLT3-ITD+ (36.4%), NPM1+ (36%), and FLT3-ITD/NPM1+ (92%) (P=0.0003). GATA-2 overexpression was also associated with WT1 (49.4% vs. 15%; P=0.005) and EVI1 overexpression (70% vs. 32.7%; P=0.004). In this series, 113 patients received induction therapy and were included in the survival analysis: 55 were male and 57 female, with a median age of 61 years. As expected, significant differences in overall survival (OS) according to age, cytogenetic group, and complete remission rate were found (P<0.01). Patients with GATA-2 overexpression had significant worse OS (P=0.036) and event free survival (P=0.039). In the stratified analysis by age, the prognostic impact of GATA-2 overexpression was particularly high in patients older than 60 years (P=0.040). We also found that OS was significantly shorter in patients with GATA-2 overexpression and no FLT3-ITD, according to the stratified analysis by FLT3-ITD mutations (P=0.022). In the same way, the prognostic impact of GATA-2 overexpression was particularly high in patients with WT1 overexpression (P=0.05). When performing the multivariate analysis, GATA-2 overexpression was an independent prognostic parameter for OS (P=0.043). In conclusion, GATA-2 overexpression is a recurrent molecular event with an unfavorable prognostic impact in AML, especially in patients older than 60 years. Advanced age is the most important prognostic factor for determining outcome in AML, therefore, it is important to identify genetic markers that could categorize cases within this subgroup. Moreover, our results show that GATA-2 deregulated expression is significantly associated with other markers of poor prognosis in AML, and could discriminate a subgroup of patients with worse prognosis in cases with NK AML and no FLT3 mutations.

0210

COMPLEX ABERRANT KARYOTYPE NEWLY DEFINED - THE STRONGEST PROGNOSTIC FACTOR IN ADVANCED CHILDHOOD MYELODYSPLASTIC SYNDROME

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Background. Karyotypic complexity has been reported to be associated with a poor or very poor prognosis. We therefore compared the outcome of children with advanced MDS identified by the traditional definition of complex karyotype, i.e. ≥3 or ≥5 chromosomal aberrations, with that of patients with a monosomal karyotype, a recently described highly unfavorable risk category in adult acute myeloid leukemia (AML). Moreover, we evaluated the prognostic significance of structural complex aberrations, a new definition of complex karyotype by the EWOG-MDS study group. *Aims.* To identify cytogenetic risk factors predicting outcome in children with advanced myelodysplastic syndrome (MDS) treated with or without hematopoietic stem cell transplantation. *Patients and Methods.* Cytogenetic analyses of 192 children prospectively enrolled in studies EWOG-MDS 98 and EWOG-MDS 2006 were centrally reviewed. Subgroups of patients with monosomy 7 or complex karyotypes (≥3 or ≥5 aberrations, monosomal karyotype, structural complex karyotype) were analyzed with respect to overall survival (OS). Structural complex constitutes a new definition of complex karyotype characterized by ≥3 chromosomal aberrations including at least one structural aberration, excluding clonal evolution of monosomy 7. *Results.* Five-year OS in patients with ≥ 3 clonal aberrations which were not structural complex did not differ from that observed in patients with monosomy 7 or a normal karyotype. Of the patients with at least two autosomal monosomies, all but one also had a structural complex karyotype. A monosomal karyotype with at least one autosomal monosomy and one or more structural aberrations that did not fulfill the criteria of structural complex did not predict an unfavorable prognosis. Cox regression analysis revealed the presence of a monosomal and structural complex karyotype to be strongly associated with poor prognosis (Hazard Ratio, HR 4.6, P <0.01). Notably, a structural complex karyotype without a monosomy was associated with a very short 2-year OS probability of 14% (HR 14.5; P <0.01). *Conclusions.* The presence of a structural complex karyotype was the strongest independent prognostic marker predicting poor outcome in children with advanced MDS.

0211

ESTABLISHMENT OF THE 1ST WORLD HEALTH ORGANIZATION INTERNATIONAL GENETIC REFERENCE PANEL FOR QUANTITATION OF BCR-ABL MRNA

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Background. Estimation of BCR-ABL mRNA levels is an important indicator of therapeutic response for patients with chronic myeloid leukemia and Philadelphia-chromosome positive acute lymphoblastic leukemia, but there is substantial variation in the real time quantitative PCR (RQ-PCR) methodology employed, what control gene is used for normalisation and how results are reported. An international scale (IS) has recently been established that is anchored to two key points defined in the IRIS trial: a common baseline (100% BCR-ABLIS) and major molecular response (0.1% BCR-ABLIS). To help propagate and improve the accessibility of the IS we sought to develop internationally accredited BCR-ABL reference reagents. *Aims.* Following an evaluation of candidate cell lines, the aim of the international collaborative study was to manufacture and evaluate a reference material panel comprising four different dilution levels of freeze dried preparations of K562 cells diluted in HL60 cells and assign fixed% BCR-ABL / control gene IS values to each material. *Methods.* Development of the materials was co-ordi-

nated by the UK National Genetics Reference Laboratory (Wessex) in conjunction with the National Institute for Biological Standards and Control (NIBSC). Four dilutions of K562 in HL60 corresponding approximately to 10%IS (BCR/ABL 4 08/198), 1%IS (BCR/ABL 3 08/196), 0.1%IS (BCR/ABL 2 08/194) and 0.01%IS (BCR/ABL 1 08/192) BCR-ABL / ABL were prepared. 3 mL glass ampoules were filled with 1.5 x 106 cells (0.5ml) for freeze drying and approximately 3500 vials of each material were produced. Field trial evaluation of the reagents was performed by 10 laboratories with stable and validated conversion factors (6 European Union, 1 Canada, 3 Asia/Australasia). *Results.* The mean amount of total RNA extracted from the reference material panel was 15.1 µg. The coefficient of variation (CV) of%BCR-ABL / control gene for each reference material indicated that the variability between ampoules was no greater than the variability of the assay on patient samples. Accelerated degradation studies showed that after 10 months of storage there has been a reduction in the BCR-ABL/control gene transcript ratio (the critical unit of minimal residual disease measurement) for ampoules maintained +56°C and +45°C but that at +37°C or below the material was stable. The mean values assigned to the reference materials following conversion to the IS are%BCR-ABL/ABL; 0.0118, 0.1112, 1.1672 and 10.7469,%BCR-ABL/BCR; 0.0195, 0.1753, 1.6627 and 16.3129 and%BCR-ABL/GUSB; 0.0071, 0.0749, 0.8295 and 10.1235. *Summary.* This study indicated that the freeze dried materials were suitable for use as reference materials for the quantitation of BCR-ABL mRNA (b3a2 transcript) using RQ-PCR. The materials were approved as the 1st International Genetic Reference Panel quantitation of BCR-ABL translocation by RQ-PCR by the Expert Committee on Biological Standardization of the World Health Organization in November 2009 and are available from NIBSC (www.nibsc.ac.uk) for the calibration of secondary reference reagents.

0212

HETEROGENEITY OF FAMILIAL HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

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Background. Familial Hemophagocytic Lymphohistiocytosis (FHL) is a rare immune deficiency with uncontrolled inflammation, characterized by fever, hepatosplenomegaly, cytopenia and hemophagocytosis. A similar clinical picture may result from mutations in different genes involved in the cellular cytotoxicity machinery, which function is abolished or severely reduced in the majority of patients. Several disease-related genes have been identified over the last 10 years: PRF1 (FHL2, OMIM 603553), UNC13D (FHL3, OMIM 608898), STX11 (FHL4, OMIM 603552). A fourth gene, STXBP2, was identified very recently as responsible for a defect of Munc18-2 (FHL5, OMIM 613101). A very similar picture has been reported also in patients with other genetic defects, including X-linked lymphoproliferative disease (XLP1, OMIM 308240), and XIAP (XLP2, OMIM 300635), Griscelli Syndrome (GS, OMIM 607624) due to RAB27A mutation, Chédiak-Higashi Syndrome (CHS, OMIM 214500) due to LYST mutation, CATCH22 Syndrome (OMIM 188400) and lysinuric protein intolerance (LPI, OMIM 222700) due to SLC7A7 mutation. *Aims.* We report the experience of the FHL Registry over 20 years (Aricò M. Leukemia 1996). *Methods.* A total of 532 patients have been reported to the Registry with suspected FHL according to the diagnostic criteria established by the Histiocyte Society. A genetic study was performed on 352 cases by direct sequencing of currently known FHL related genes. *Results.* In 143 patients a genetic marker was identified. They were reported from the following countries: Italy, n=113; England, n=14; Uruguay, n=4; USA, n=4; Spain, n=3; Germany, n=2; France, n=1; Belgium, n=1; Colombia, n=1. The genetic diagnoses were as follows: FHL3 n=62 (43%), FHL2 n=58 (41%), XLP n=8 (6%), FHL5 n=7 (5%), GS n=3, CHS n=2, LPI n=2, CATCH22 n=1. Although the clinical picture is similar in FHL2 and FHL3, the median age at diagnosis was younger in FHL2 (median 2.3 months) than in FHL3 (5.3 months). It is remarkable that one quarter of the patients was diagnosed when older than 4.5 years, the older patient being 27-year old. *Conclusions.* Current diagnostic approach is based on clinical criteria and flow-cytometry for perforin expression and granule release assay, and may be supported by cytotoxicity assay. Mutation analysis is mandatory for identification of the familial marker. Prevalence of FHL3 appears to be comparable to that of FHL2, with FHL5 accounting for a minority of cases while FHL4 was never diagnosed in this series. Based on the current knowledge, a genetic defect may be assigned to about 80% of the familial cases, thus supporting indication to hematopoietic stem cell transplantation, and allowing selection of familial donors, counselling, and family planning.

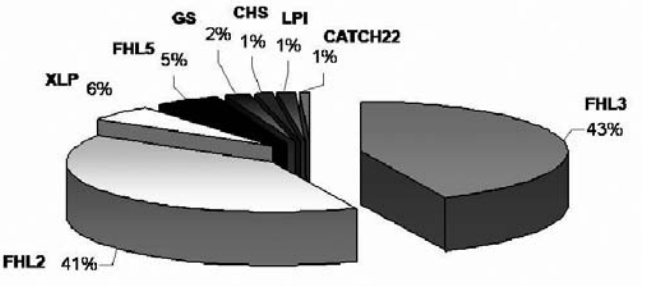

Figure. Distribution of different types of FHL.

0213

CYTOGENETIC FOLLOW-UP IN PATIENTS WITH IN MYELODYSPLASTIC SYNDROME TREATED WITH LENALIDOMIDE: IMPACT OF KARYOTYPING AND FLUORESCENCE IN SITU HYBRIDIZATION

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Background. Lenalidomide has been shown to be particularly active in MDS patients with del(5q) leading to transfusion independence in over two-thirds of patients with low and intermediate risk MDS and 5q deletion. Patients without a cytogenetic response had a significantly increased risk of progression. Therefore, careful monitoring of the cytogenetic response is extremely important in all MDS patients treated with lenalidomide. *Aims.* In order to optimize therapy control of MDS patients treated with lenalidomide, we have reviewed the cytogenetic data of patients treated with lenalidomide within the MDS-003 and MDS-004 study and compared the results obtained by different methods like karyotyping and FISH. *Methods.* Our cohort included 302 patients with transfusion-dependent anemia due to low- or intermediate-1-risk myelodysplastic syndrome associated with a 5q deletion with or without additional cytogenetic abnormalities. Patients were enrolled in the studies MDS-003 (n=42) or MDS-004 (n=260). Cytogenetic and molecular cytogenetic investigations were performed according to standard procedures. Whenever possible, 25 metaphases obtained from 24-hour and in some cases also from 48-hour cultures were analysed. Fluorescence *in situ* hybridization (FISH) for deletion 5q was included in each investigation. *Results.* We have reviewed 1075 cytogenetic and FISH analyses of 302 MDS patients with del(5q) treated with lenalidomide. A complete cytogenetic investigation based on at least 25 metaphases and FISH based on the analyses of at least 200 interphase nuclei was made in 835 (78%) of the investigations. The median detection rate of a deletion in 5q in metaphases and interphases was 54% and 33%, respectively (P<0.05). Overall, classical banding analysis was significantly more sensitive in detecting a deletion 5q. In 19 patients and in 39 investigations, del(5q) was detected by karyotyping only. Thus, a cytogenetic non-response or relapse of the disease would have been missed if only FISH analyses would have been performed. In 8 patients undergoing a karyotypic evolution, and in 17 investigations neither the del(5q) nor additional chromosome aberrations were detected by FISH - in contrast to karyotyping. *Conclusions.* Classical banding analysis was significantly more sensitive in detecting a deletion 5q than FISH. This result can be explained by a proliferative advantage of cells with a deletion 5q. In conclusion, to optimize the treatment strategy and therapy control of patients treated with lenalidomide and identify cytogenetic non-response or progressions as early as possible, both classical banding analysis and FISH should be performed in follow-up investigations.

0214

IDENTIFICATION OF A CONSTITUTIONAL T(16;21) INVOLVING 16P13 ATF7IP2 AND 21Q22 RUNX1 LOCI AS A NOVEL GENETIC MECHANISM IN FPD/AML

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Familial platelet disorder with propensity to myeloid malignancy (FPD/AML) is an autosomal dominant disorder characterized by platelet abnormalities and a predisposition to AML. FPD/AML is caused by inherited mutations of 21q22 *RUNX1*. Given the clinical heterogeneity of FPD/AML, the syndrome could be overlooked, and has resulted in SCT of patients from affected siblings. Recently, we and others reported on constitutional *RUNX1* copy number changes that would go unnoticed by sequencing, as a second pathogenomic mechanism in FPD/AML. We therefore have implemented array CGH and FISH for *RUNX1* in diagnosing three FPD/AML families. We present the identification of two novel *RUNX1* mutations by sequencing (G143R and Q235X). In a third family no *RUNX1* abnormality was detected in the proband by sequencing or array CGH on AML bone marrow. However, by FISH we identified a *RUNX1* gene rearrangement resulting from a cryptic t(16;21)(p13;q22). The constitutionality of the t(16;21) *RUNX1* rearrangement as the third pathogenomic mechanism in FPD/AML was demonstrated by FISH on cells from the urinary tract epithelium and buccal mucosa. The 16p13 breakpoint was confined by FISH to *ATF7IP2* (*MCAF1*). ATF7IP2 is a transcription factor involved in proliferation in human cancer by epigenetic transcriptional control of Sp1-dependent maintenance of telomerase activity. This is the first report on a constitutional chromosome translocation with an oncogene rearrangement as a pathogenomic mechanism in a hematological malignancy. Our data indicate that FISH for *RUNX1* has to be performed for structural anomalies along with sequencing and copy number analysis in diagnosing FPD/AML.

0215

A MUTATED NOTCH1 GENE IS PROGNOSTIC FAVORABLE IN PEDIATRIC LEUKEMIA OF T-CELL ORIGIN

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Background. Acute lymphoblastic leukemia (ALL) is the most common pediatric leukemia. ALL with T-cell origin (T- ALL) accounts for 15% of these and have a worse prognosis than ALL of B-cell origin. With intensive treatment protocols event free survival approaches 80% for T-ALL but the outcome for the remaining 20% is dismal. A major goal is therefore to find markers that can identify patients at high risk for relapse or treatment resistant disease already at diagnosis in order to offer these patients intensified or alternative treatment approaches. *Aim.* The aim of the study was to investigate genetic alterations in pediatric T-ALL and to determine their prognostic significance. *Material and Methods.* Thirty-three pediatric T-ALL samples were analyzed for the presence of mutations in the NOTCH1 and FBWX7 genes. In addition, genetic alterations were investigated with molecular allelokaryotyping (250K Nsp SNP-array), conventional and spectral karyotyping (SKY), flourescence *in situ* hybridization (FISH) and multiplex ligation-dependent probe amplification (MLPA) analysis. All T-ALL patients included in the study were diagnosed and treated at one single centre 1991-2008 according to common NOPHO ALL protocols. *Results.* Mutational analysis included analysis of two regions in the NOTCH1 gene, the heterodimerization domain (HD) and the proline/glutamic acid/serine/threonine (PEST) domain, and the FBWX7 gene. NOTCH1 mutations were found in 25 patients (76%). A double-mutation (both HD and PEST) was found in 6 patients (18%). A single HD mutation was found in 10 patients (30%), and two of these also had an FBWX7 mutation. A single PEST mutation was found in 9 patients (27%), one of these also had a FBWX7 mutation. Correlation analysis showed a significantly better disease-free survival for patients with NOTCH1 gene mutated leukemia at 5 years (57% vs. 22%, P=0.011) with a clear trend towards better overall survival (57% vs. 33%, P=0.073). All analyzed patient samples presented one or more genomic lesions in addition to NOTCH1 or FBWX7 gene mutations. Diploid or near-diploid karyotypes predominated in the material. Eight patients had an apparently normal karyotype and six cases had a complex karyotype. Three patients had balanced translocations including two t(11;14)(p13;q11) and one t(9;13)(p12;q?) translocation. Isochromosome, i(9)(q10), was seen in one case and i(17)(q10) in two cases. Molecular allelokaryotyping identified mainly genetic losses in T-ALL and 205 copy number changes (CNC) were detected, including 158 deletions, 25 gains and 22 uniparental disomies (UPD). All chromosomes but chromosome 22 and the X chromosome were affected. The most frequently affected chromosomes were 5, 7, 9, 11 and 14. Loss of 9p21.3 (p16/CDKN2A) was the most frequent aberration and detected in 80% of the cases. No significant correlation was found for any of the genetic aberrations with the presence of a NOTCH1 gene mutation or clinical outcome. The presence of a NOTCH1 gene mutation did not correlated with the level of white blood cells at diagnosis. *Conclusions.* A NOTCH1 gene mutation was found in more than 3/4 of investigated pediatric cases of T-ALL and was associated with a significantly better disease free survival.

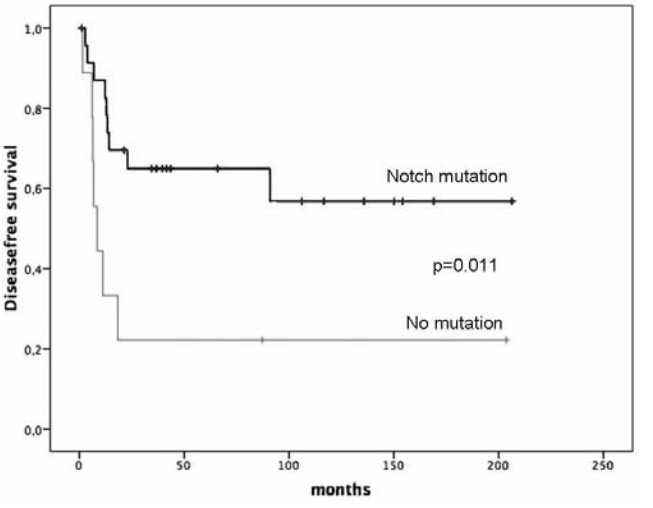

Figure. Kaplan-Meier curve of pediatric T-ALL.

0216

ORIGIN OF THE INV(16)(P13Q22) IN *DE NOVO* AND THERAPY-RELATED ACUTE MYELOID LEUKEMIA

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Background. Therapy-related acute myeloid leukemia (t-AML) may arise after cytostatic and genotoxic therapies for various malignancies. The incidence of t-AML is about 5% of all cases with AML and this subtype of leukemia is associated with low survival rates. Some drugs that are used to treat primary tumours are responsible for the generation of chromosomal translocations in t-AML. For instance, topoisomerase II inhibitors may trigger the formation of chromosomal translocations that play a causal role in the development of t-AML. This assumption is based on the fact that genomic translocation breakpoints observed in patients with t-AML cluster near topoisomerase cleavage sites while breakpoints in *de novo* AML are randomly distributed over large areas. *Aims/Methods.* The chromosomal rearrangement inv(16)(p13q22) is found in both *de novo* and t-AML. This aberration results in a CBFB-MYH11 fusion gene that plays an essential role in leukemogenesis. To gain insight into the mechanisms causing the inv(16) we characterized genomic CBFB and MYH11 breakpoints from 29 cases with *de novo* AML and compared these to 6 cases with t-AML. Primary tumours observed in patients with t-AML included mammary (n=2), cervix (n=2) and ovarian carcinomas (n=1) and acute lymphoblastic leukemia (n=1). The latency between the initial diagnosis and the development of t-AML varied from 2 to 6 years. Treatment regimens for primary tumours included cyclophosphamide, fluorouracil, methotrexate, carboplatin, etoposide, tamoxifen, epirubicin, cisplating and radiation. Genomic breakpoints were characterized by direct sequencing of obtained PCR products. *Results.* We observed that genomic CBFB and MYH11 breakpoints in *de novo* AML were random-

ly distributed. Importantly, the genomic breakpoints in patients with t-AML did not cluster. In fact, the distribution was very reminiscent of that observed in *de novo* AML. Because some patients with t-AML were originally treated with topoisomerase inhibitors we studied whether topoisomerase inhibition resulted in double strand DNA breaks in CBFB and MYH11. While the topoisomerase inhibitor etoposide readily induced genomic breakpoints in the MLL locus, we did not observe these in the relevant CBFB and MYH11 regions in leukemia cell lines. *Discussion*. These data may suggest that the inv(16) in t-AML and *de novo* AML are induced by a common mechanism but that cytostatic and genotoxic drugs as used in cancer therapy are not implicated in this process. It is well known that the CBFB-MYH11 fusion is essential for leukemogenesis but other mutations are required for the development of full blown leukemia. Thus, cytostatic and genotoxic drugs may induce mutations that together with the inv(16) result in therapy-related AML.

0217

HIGHER PREVALENCE OF ADVERSE PROGNOSTIC GENETIC ABERRATIONS AND IGH-MUTATION STATUS IN PATIENTS WITH SMALL LYMPHOCYTIC LYMPHOMA AS COMPARED TO CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL) are considered the same entity. It has been convincingly shown that the clinical course and the overall survival of patients with chronic lymphocytic leukemia (CLL) largely depend on molecular parameters, such as specific genetic abnormalities, mutational status of the variable region of the immunoglobulin heavy chain genes (IGHV) and the usage of specific VH genes. However, knowledge on mutation status and genomic aberrations on diagnostic specimens from SLL patients have hardly been reported in the literature. *Aim.* The prevalence of molecular prognostic parameters in classical SLL was compared to those in CLL with lymph node involvement and to CLL without lymph node involvement. *Methods.* Genomic DNAs extracted from lymph nodes or peripheral blood cells of patients with SLL (n=22), patients with lymphocytosis and affected lymph nodes (n=16) and patients with B-CLL (n=82) were analyzed for IGH-mutation status and VH-family usage. Chromosome aberrations such as 11q-, +12, 13q- and 17p- were determined by multiplex ligation dependent probe amplification (MLPA). *Results.* The majority of lymph node samples from SLL patients (64%) showed unmutated IGH-variable gene segments, which was significantly higher than in the samples of CLL patients (30%). In contrast, high-risk chromosome abnormalities (11q-, 17p-, +12) were more prevalent in patients with lymph node involvement (SLL + CLL; 48%) as compared to CLL patients without (21%). After combining the VH-mutation and chromosome aberration data, 73% of the SLL patients exhibited adverse prognostic factors, which is also significantly higher (43%) as compared to CLL patients without lymph node involvement. CLL patients with lymph node involvement exhibited almost equal frequencies of poor risk chromosome aberrations (50%), but lower frequencies of unmutated IGH genes (38%), as compared to patients with SLL. Interestingly, usage of the VH3-21 gene was not found in patients with SLL or CLL with lymph node involvement. Usage of the VH1-69 gene (all unmutated) was frequently found in patients with SLL (27%), of which half of the cases exhibited adverse chromosome aberrations as well. *Conclusions.* SLL patients show a higher occurrence of adverse prognostic molecular genetic parameters as compared to CLL patients. For optimal patient care, we recommend integrated assessment of VH-gene mutational status and chromosome aberrations on the same diagnostic specimen.

0218

CHROMOSOMAL ABNORMALITIES IN TRANSFORMED PH-NEGATIVE MYELOPROLIFERATIVE NEOPLASMS (MPN) ARE ASSOCIATED TO THE TRANSFORMATION SUBTYPE AND INDEPENDENT OF THE JAK2 AND THE TET2 MUTATIONS.

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Evolution to myelofibrosis (MF), acute myeloid leukemia or myelodysplastic syndrome (AML/MDS) may occur over time in MPN patients most likely due to the acquisition of additional mutations. The Groupe Francophone de Cytogenetique Hematologique has collected and reviewed 82 patients with transformation of MPN (66 AML/MDS and 16 MF). All patients gave informed consent in agreement with the Helsinki declaration. JAK2V617F and TET2 mutations were searched for in 40 and 32 patients, respectively. Significantly more -7/del(7q) (P=0.004) and -5/del(5q) (P=0.03) were found in AML/MDS with a higher incidence of dup1q (P=0.01) and trisomy 9 (P=0.04) in MF. Some specific chromosomal abnormalities occurred together, for example -5/del(5q) and -17/del(17p) (P=0.0007). In multivariate analysis, two factors were independently associated with an inferior overall survival (OS); AML/MDS transformation (P<0.0001) and -5/del(5q) abnormality (P=0.02). Although both giving rise to loss of 7q, der(1;7) differed from other 7q deletions in terms of distribution (lower frequency of AML/MDS, P=0.02), association with chromosomal abnormalities (absence of -5/del(5q), P=0.003; increased del(20q), P=0.05), and longer OS (P=0.0007). We detected 24/40 (60%) JAK2V617F and 8/25 (32%) TET2 mutations in samples following transformation, ranging from wild-type to mutated forms of both genes. The mutated and wild-type forms of the genes were not found to be associated with a specific chromosomal abnormality. There was no evidence that JAK2 or TET2 mutations were associated with the type of MPN transformation, whereas the type of cytogenetic abnormalities were strongly linked, perhaps indicating that they play a specific role in the transformation process. The observation that certain chromosomal changes were found together suggested an oncogenic cooperation between them.

0219

DYSREGULATION OF MIR-92A, AS A POSSIBLE APOPTOSIS INHIBITOR, INDICATES POOR PROGNOSIS IN ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. The miR-17-92 cluster is a polycistronic miRNA gene, encoding six miRNAs (miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92), and several studies have suggested the importance of the miR-17-92 cluster in immune cell development, as well as in lymphomagenesis. Recently, we have shown that the down-regulation of miR-92a in plasma is a novel biomarker in human leukemia (Tanaka et al, 2009, PLoS One 4(5): e5532), however, the precise mechanisms of miR-92a dysregulation in leukemia cells have not been fully elucidated. *Aims.* The aim of this study was to determine plasma and cellular miR-92a expression levels in acute leukemia patients, in order to clarify whether or nor it could be an independent prognostic factor, and to establish a novel therapeutic strategy using miR-92a in acute leukemia. *Methods.* Total RNA was isolated from both mononuclear cells and plasma obtained from 91 acute leukemia patients. This study was approved by the institutional review board of Tokyo Medical University (no. 930: approved on June 24, 2008). Written informed consent was obtained from all patients according to the Declaration of Helsinki prior to collection of the specimens. Expression of miR-92a was quantified using TaqMan® MicroRNA assays using 2-ΔΔCt *Methods.* The miR-92a expression was normalized to U6B or miR-638 expression, yielding a -

ΔCt value. *Results.* In healthy volunteers, the miR-92a expression level in cells ranged from 0.92 to 1.62 (mean±SD, 1.26±0.23) and those in plasma varied from 0.22 to 3.58 (mean±SD, 1.2±0.92). The miR-92a expression level in plasma was significantly lower, approximately one hundredth, in both acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) compared to normal controls (P<0.0001). Unlike miR-92a expression in plasma, there was a considerable variation of miR-92a expression in acute leukemia cells: the miR-92a expression level varied from 0.12 to 4.72 (mean±SD; 1.01±1.05) in AML, and it ranged from 0.22 to 10.00 (mean±SD; 2.23±2.34) in ALL. The miR-92a expression level was significantly higher in ALL cells compared to normal controls (P=0.0272), while there was no significant difference between AML cells and normal controls (P=0.3848) . Notabley, ALL patients with over-expressed miR-92a had poor outcomes (P=0.0186). The knock-down of miR-92a by antisense oligonucleotide revealed an increase of apoptotic cells in Raji and OM9;22 cells, indicating that miR-92a may contribute as a possible apoptosis inhibitor in a subset of ALL. *Conclusions.* Although questions still remain about the dynamics of miR-92a in leukemia cells, our results highlight the dysregulation of miR-92a in acute leukemia cells. Our findings suggest that the quantification of miR-92a in leukemia cells can be used as a prognostic marker in ALL, and it can be used for monitoring leukemia patients together with measurement of plasma miR-92a in both ALL and AML patients.

0220

ABNORMAL EXPRESSION OF MIR-9 INDICATES POOR PROGNOSIS IN AML PATIENTS

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*Background.*miRNAs are small non-coding RNAs that suppress protein translation by interacting with 3' untranslated regions (UTR) of target mRNAs. Recent studies suggest that abnormal expression of miRNAs contributes to the development of various cancers, including hematological malignancies. RUNX1 is a Runt family transcription factor critical for normal hematopoiesis. Inactivation of RUNX1 function through chromosomal translocations or gene mutations results in the development of acute myelogenous leukemia (AML) and myelodysplastic syndrome. We speculate that aberrant expression of miRNAs negatively regulating RUNX1 mRNA could be another underlying molecular basis in suppressing RUNX1 function. [Aims] To test the above hypothesis with the samples of AML patients, we first evaluated expression levels of miRNA whose seed sequences reside within the 3' UTR of human RUNX1 mRNA (referred as RUNX1-related miRNA). Secondly, we analyzed the relationship between its abnormal expression and clinical features. *Methods.* Bone marrow samples were collected from 87 AML patients at diagnosis (FAB-M0, 7; M1, 11; M2, 29; M3, 13; M4, 10; M5, 10; M6, 6; and M7, 1) and 23 healthy controls at Dokkyo Medical University Hospital between 2000 and 2009. This study was approved by ethical committee of our institute, and written informed consents were provided by all of the patients. Total RNAs were isolated with the mirVana miRNA Isolation kit (Applied Biosystems), and Taqman MicroRNA Assays (Applied Biosystems), was used to quantify the RUNX1-related miRNAs including miR-9, miR-18a, miR-27a, miR-27b, miR-30a-5p, miR-30b, miR-30c, miR-30d, miR-30e-5p and miR-199a. Differences in overall survival (OS) and relapse-free survival (RFS) were analyzed with the Kaplan-Meier method, and both univariate and multivariate statistical analysis were performed using SPSS software. *Results*. Among the RUNX1-related miRNA, about 15% of AML patients expressed miR-9 that was barely detected in other AML patients and the controls. We divided the AML patients into two groups, miR-9$^+$ group (n=16) and miR-9(-) group (n=71), based on the expression level of miR-9. Between these two groups, there were no significant differences in known AML prognostic factors such as age, white blood cell count at diagnosis, and FLT3-ITD or FLT3-TKD mutations. In Kaplan-Meier analysis, miR-9$^+$ group exhibited significantly inferior OS and RFS compared to miR-9$^-$ group. Because complete remission rate was comparable between these two groups, these results suggest that miR-9$^+$ group were more likely to relapse than miR-9(-) group. Univariate and multivariate analysis showed that expression of miR-9 was an independent prognostic predictor for both OS and RFS. *Summary/conclusions*. We analyzed expression levels of RUNX1-related miRNA which could potentially suppress protein expression of RUNX1 in a AML patient cohort, and found that miR-9 was specifically expressed in a part of these patients. Because aberrant expression of miR-9 emerged as a strong negative prognostic factor, there could be a causative relationship between the aberrant expression and leukemogenesis. To fully understand the molecular pathogenesis in miR-9-overexpressing leukemia, we need to identify other targets of miR-9 than RUNX1 mRNA.

0221

MOLECULAR CHARACTERIZATION OF A NOVEL CHROMOSOMAL TRANSLOCATION T(12;14)(Q23;Q11.2) IN T-LYMPHOBLASTIC LYMPHOMA BETWEEN THE T CELL RECEPTOR DELTA DELETING ELEMENTS AND THE HYPOTHETICAL GENE C12ORF42

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Chromosomal aberrations have diagnostic, prognostic and therapeutic consequences in haematological malignancies. By combining fine-tiling comparative genomic hybridization (FT-CGH) and ligation-mediated PCR (LM-PCR) we established a fast and robust approach to precisely characterize chromosomal breakpoints. Using this approach we characterized at the molecular level novel chromosomal translocation t(12;14)(q23;q11.2) in T-lymphoblastic lymphoma occurring during the deletional rearrangement of the T cell receptor delta gene (TRD), which normally is a pivotal step in T cell differentiation towards the alpha/beta versus the gamma/delta lineage and generates the T cell receptor excision circles (TREC) used to determine the proliferative history of T cells. We found that this rearrangement disrupted the hypothetical gene C12orf42, and brought into proximity to the TRA enhancer the Achaete-scute complex homolog 1 (ASCL1) gene, which encodes a member of the basic helix-loop-helix (BHLH) family of transcription factors and is overexpressed in medullary thyroid cancer and small cell lung cancer. This broad applicable method for detailed molecular analysis of chromosomal abnormalities shall facilitate the identification of new target genes for molecular therapy of malignant diseases.

0222

EVALUATION OF CYTOGENETICS WHOLE-GENOME 2.7M ARRAY AS A NEW CLINICAL TOOL FOR DETECTING CYTOGENETIC ABNORMALITIES FOR CHRONIC LYMPHOCYTIC LEUKEMIA PROGNOSIS. A COMPARISON WITH INTERPHASE FISH

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Background. Chronic lymphocytic leukemia (CLL) is a hematologic malignancy with a variable clinical course. Many CLL prognostic factors have been defined. Among them, cytogenetic abnormalities such as del(17p) and del(11q) have been tightly related to progression and shorter overall survival. In CLL, the most recurrent cytogenetic abnormalities are del(13q), del(11q), trisomy 12, del(17p) and del(6q), detected in up to 60-70% of patients by interphase fluorescence *in situ* hybridization (iFISH). Recently, array-based karyotyping has gained acceptance as a new tool for detecting genomic imbalances with higher resolution and better cost/benefit ratio than other techniques used nowadays. *Aims.* To analyze cytogenetic alterations in CLL using a newly developed Cytogenetics Whole-Genome 2.7M (Affymetrix). To identify recurrent or non- previously defined prognostically important aberrations in CLL. To compare array results with G-banding cytogenetics and iFISH and correlate them with clinical and molecular data to test array-CGH as a clinical tool in CLL. *Methods.* Eighteen patients with CLL were included in the study (11M/7F, median age 74) previous to any treatment. DNA was extracted from whole peripheral blood and submitted to Cytogenetics Whole-Genome 2.7M array. All patients were also studied by G-banding cytogenetics and iFISH with the classical CLL panel (11q22.3, CEP 12, 13q14 and 17p13 probes). *Results.* FISH analysis detected recurrent aberrations in 11/18 (61.1%). The most common alteration was del(13q) (eight patients, 44%), followed by trisomy 12 (four patients, 22%). Del(17p) and del(11q) were found in one patient each as unique deletion (5.6%). Whole Genome 2.7M array could detect aberrations tested by iFISH probes in a total of eight patients. Losses in 13q were the most frequently detected (5 cases, 27%), trisomy 12 was detected in four (22%) and del(11q) in a single

patient. Losses found in 13q14 cytoband ranged from 111 to 2940 Kb and the minimal common deleted region size was 11Kb (49522757-49634282). Only one deletion contained the region where miR15 and miR16 were located. Array-CGH results were concordant with iFISH in 15 patients but failed to detect del(13q) in three cases and del(17p) in one patient. Samples with deletions missed by array-CGH had been detected in less than 25% of cells analyzed by iFISH. Contrarily, array-CGH did not detect any abnormality missed by iFISH probes. Additional gains and losses were identified in 14 patients. A deletion of 5q32-q34 in one case, and trisomies for chromosomes 18 and 19 had already been detected by G-banding cytogenetics. However, gains of full length 2p and 22q arms and other smaller abnormalities had not been identified by conventional techniques. *Conclusions.* Recurrent aberrations with prognostic impact have been identified in more patients using iFISH techniques. Cut-off values for Whole Genome 2.7M sensitivity have found to be 25% due to low tumoral infiltration. Array-CGH allowed to detect other alterations with unknown clinical significance, some of them previously detected by G-banding cytogenetics. These results are preliminar. Additional patients should be analysed to demonstrate the Whole Genome 2.7M applicability in clinical practise for CLL. *Acknowledgements*. Red Temática de Investigación Cooperativa en Cáncer No. RD07/0020/2004.

0223

GENOTYPE-PHENOTYPE STUDY OF FAMILIAL HAEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS DUE TO UNC13D MUTATIONS

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Background. Familial Hemophagocytic Lymphohistiocytosis (FHL) is a genetically heterogeneous disorder characterized by fever, hepatosplenomegaly, cytopenia and frequent central nervous system involvement. In most cases the natural course is rapidly fatal unless appropriate treatment is applied. Since 2003, mutations of UNC13D, the gene encoding Munc 13-4, a protein essential for cytolytic granule fusion to the cell surface membrane, have been recognized as the cause for FHL3 (OMIM 608898). Previously published reports on FHL are based on limited cohorts of patients from single centres, with the only exception of a recent genotype-phenotype study of FHL2. *Aims.* The aim of the present study is to analyze genotype-phenotype correlations in a large cohort of patients with FHL3. *Methods.* The consortium established between Italy, Germany and Sweden planned to pool in a common database data on presenting features, mutations, and cytotoxic function from individual patients with FHL3 diagnosed on the basis of documented biallelic UNC13D mutations. Statistical analysis was performed by SPSS 11.5 software; P<0.05 was considered as significant. *Results.* a total of 83 patients (46 male, 37 female; median age: 4,4 months) with FHL3 were reported from the following centres: Florence, Italy (n=54), Hamburg, Germany (n=16), Stockholm, Sweden (n=13). They had been diagnosed between 1981 and 2009. The ethnic origin was as follows: Caucasian, n=57, Turkish, n=8, Asian, n=7, Hispanic, n=4 (not reported, n=4). Regarding diagnostic criteria, thrombocytopenia was present in 96%, splenomegaly in 95%, fever in 89%, anaemia in 86%. Central nervous system involvement was present in 50/80 (62%) patients, a proportion which is significantly higher than in FHL2 (n=31/86; 36%; P=0.001). Granule release capacity detected by CD107a expression, NK activity and Munc 13-4 protein expression were absent or reduced in all patients analyzed with the exception of a single patient with an atypical phenotype and compound heterozygocity for a missense and a frameshift mutations. 53 different mutations were observed, including 15 novel ones: 17 missense, 14 frameshift, 12 nonsense and 10 splice errors. No private mutations for specific ethnic groups were found. To explore the correlation between specific mutations and age at diagnosis, we grouped the patients according to the functional impact of the mutations; the groups were defined as two disruptive (nonsense, frameshift and selected splice errors) mutations, two missense mutations, and a third group including compound heterozygous with one missense and one disruptive mutation. Patients with disruptive mutations had a significantly younger age at onset (P=0.001). *Conclusions.* UNC13D mutations are scattered over the entire gene, without hot spots. Despite different ethnic and geographic origin of the patients, there are apparently no ethnic-specific mutations. The clinical picture of FHL3 is comparable to that of FHL2, although CNS involvement appears to be more frequent. The granule release assay appears to be a very sensitive tool for screening of FHL3.

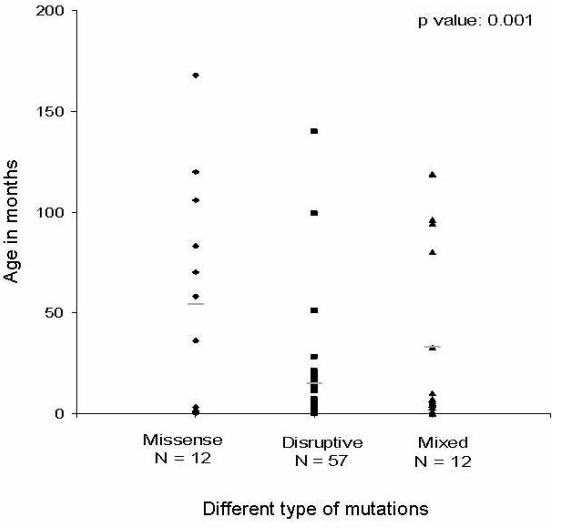

Figure. Different types of mutations and age of onset

0224

GENOMIC PROFILING USING SNP-BASED MICROARRAYS IN A DIAGNOSTIC SETTING FOR THE IDENTIFICATION CHROMOSOMAL ABNORMALITIES IN ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. Gross cytogenetic anomalies are being used as diagnostic, prognostic and therapeutic markers in the clinical management of acute lymphoblastic leukemia (ALL). Recently, it has become clear that ALL cells frequently harbor relevant disease-related submicroscopic chromosomal abnormalities. Although karyotyping is generally considered as the gold standard in the genetic diagnosis of ALL, this method is limited by its capacity to detect only those copy number changes that are microscopically visible (>5-10 Mb in size). In contrast, SNP-based microarray analyses allow a genome-wide detection of copy number changes less than 100 kb in size. Such analyses also overcome some of the other major limitations of karyotyping such as low success rates due to inadequate metaphase yield and/or poor banding quality. *Aim.* To investigate whether microarray-based genomic profiling is applicable in a routine diagnostic setting. *Methods.* For our analyses we used a 250k SNP microarray platform. In order to rule out irrelevant (benign) copy number variations (i) thresholds for copy number aberrations were set at >5 Mb and (ii) smaller gains or losses were interpreted as aberrant only in case they coincided with known (recurrent) aberrations as reported in the literature or in the "Atlas of Genetics and Cytogenetics in Oncology and Haematology"(http://atlasgeneticsoncology.org/). *Results.* Cytogenetic, FISH and SNP-based microarray data of 60 diagnostic ALL samples were compared. Through microarray analysis, almost all numerical abnormalities as found by karyotyping were detected. More importantly, in the majority of samples additional small recurrent abnormalities with a known prognostic relevance were detected. As expected, balanced chromosomal abnormalities were not identified. Therefore, it remains recommended to apply FISH and/or PCR-based methods for the detection of translocations with a known diagnostic/prognostic impact (e.g. BCR-ABL1) in addition to microarray analysis. *Conclusions.* We demonstrate that SNP-based microarray analysis serves as a comprehensive and reliable diagnostic tool for the identification of clinically relevant chromosome aberrations in ALL.

0225

UNINTENTIONAL 'STEM CELL TRANSPLANT' FOLLOWING A LIVER TRANSPLANT IN A PATIENT WITH MONOSOMY 7 AML

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A 16 year old female developed 100% hematopoietic donor chimerism from a female liver transplant donor. She initially received a conventional sibling stem cell transplant (SCT) for monosomy 7 AML in January 2008. By April 2008 she had relapsed and following chemotherapy, received a second, matched unrelated male donor SCT in August 2008. Full donor chimerism was achieved. In November 2008 she developed graft versus host disease (GVHD) resulting in liver failure, and had a liver transplant. Within 3 weeks of the liver transplant, the male second donor chimerism level fell to 3% after a period of profound neutropenia, with no evidence of AML relapse. Chimerism analysis revealed the appearance of DNA of multiple unknown origins, with one unknown origin subsequently becoming dominant. Further investigation identified the origin as the female liver donor. Over an 8 week period full engraftment of liver donor hematopoiesis occurred and persists 9 months after the transplant with no GVHD, despite complete HLA mismatch. Engraftment of passenger hematopoiesis following solid organ transplant has been described previously, but is exceptionally rare, including a case of complete hematopoietic chimerism and tolerance of a liver allograft from a male donor in a 9 year old girl.

0226

DETECTION OF RARE COPIES OF BCR-ABL1 TRANSCRIPT IN PATIENTS WITH PHILADELPHIA POSITIVE (PH+) ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) WITH A HIGH SENSITIVE NANOFLUIDIC ARRAY

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Background. Ph+ ALL is observed in about 20-30% of adult ALL and is associated with a very poor prognosis and early relapse. Tyrosine kinase inhibitors have improved overall treatment results, with a rapid response and a complete remission (CR) rate ranging 90%. Nevertheless most patients experienced hematological relapse in a short time, also after hematopoietic stem cell transplantation. Molecular analysis based on quantitative assays (i.e. quantitative polymerase chain reaction, qPCR) provides detection of residual leukemic cells measuring BCR-ABL1 transcript level and becomes necessary in the monitoring of minimal residual disease to confirm molecular CR and to detect early relapse. *Aim.* To investigate the efficacy of a high sensitive method based on nanofluidic platform (Fluidigm Corporation, South San Francisco, CA) to detect and quantify residual and rare BCR-ABL1 copies in Ph+ ALL patients who obtained molecular remission as assessed by conventional qPCR. *Methods and Patients.* The 12.765 Digital array (Fluidigm) is a nanofluidic biochip that consists in twelve panels, each containing 765 individual reaction chambers of 6 nL volume. Samples are portioned prior to qPCR into the single chambers of the panel; as fluorescent signal is produced only in chambers containing copies of the target sequence, digital array provides an absolute quantification by counting the number of positive reactions. Following amplification, digital raw data are processed by the BioMark Digital PCR Analysis software (Fluidigm), that estimates the true number of molecules per chamber using the Poisson probabilistic distribution. At the time of writing, we analyzed 22 Ph+ ALL samples (11 positive for the P190 BCR-ABL1 isoform and 11 for the P210) who were in complete (87%) or major (13%) molecular response (BCR-ABL1/ABL ratio ≤0.001 or <0.1, respectively) as assessed by conventional qPCR; RNA integrity was evaluated using the control gene ABL. *Results.* First, we assessed the sensitivity and reproducibility of the assay using six serial dilutions of plasmids (Ipsogen) expressing known copy number of BCR-ABL1 P190 transcript (10000; 1000; 100; 50; 10; 1 copies). A 2 µL volume of input cDNA was loaded and two panel for each dilution were used. Analysis parameter chosen for digital raw data processing were automated set threshold of 0.65 and target Ct range 20-40. Results showed a detection rate until a copy of target sequence and a pairing significantly effective between replicates (P=0.0014, Paired t TEST analysis). We then analyzed duplicates of Ph+ ALL samples with a positive control for each chip: digital array resulted positive in 58% of complete molecular response samples, with 5.5 as median number of copies detected (range 0.5-11). *Conclusions.* The Fluidigm nanofluidic platform provides a high sensitive assay, able to detect until a single copy of BCR-ABL1 transcript with greater accuracy than conventional qPCR, as demonstrated for samples in molecular remission, and could provide an accurate monitoring method for Ph+ ALL CR patients. Further studies to confirm these results are actually ongoing. Supported by European LeukemiaNet, AIL, AIRC, FIRB 2006, Ateneo RFO grants, Project of integreted program (PIO), Programma di Ricerca Regione - Università 2007 - 2009.

0227

DIAGNOSING CML - DETECTION OF BCR-ABL1 PROTEIN BY FLOW CYTOMETRY

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Background. In investigating patients with neutrophilia and/or thrombocytosis, it is important to exclude chronic myeloid leukaemia (CML). Definitive diagnosis of CML requires detection of either the t(9;22) Philadelphia chromosome translocation (by metaphase spreads or FISH), or the fusion gene BCR-ABL1 by PCR. These techniques however are usually confined to specialist regional laboratories, are costly and may take valuable time to complete. Since most patients with neutrophilia and/or thrombocytosis do not have CML, a rapid and simple screening test for CML would be quite valuable. This could be achieved by the demonstration of the expression of the BCR-ABL1 protein, but this has hitherto been technically difficult because of the lack of a specific antibody that could reliably distinguish BCR-ABL1 protein. *Aims.* We investigated if the newly developed BCR-ABL protein kit (BD Biosciences) could accurately identify BCR-ABL1 fusion protein and be used as a rapid screening test for the identification of CML patients. *Methods*. Peripheral blood samples from known CML patients, and patients with neutrophilia and/or thrombocytosis suspected of having CML (n =75) were collected, as well as normal healthy controls (n=22). Each sample was analysed for BCR-ABL1 translocation both by PCR and with the BCR-ABL protein kit. For the BCR-ABL protein kit, cells were lysed; lysates were first incubated with BCR-antibody-coated capture beads (recognising an epitope upstream of BCR at the 5' end of the m-bcr region) and subsequently with PE-labelled detector ABL antibodies (recognising an ABL epitope downstream of exon 3). Samples were analysed by standard flow cytometry.

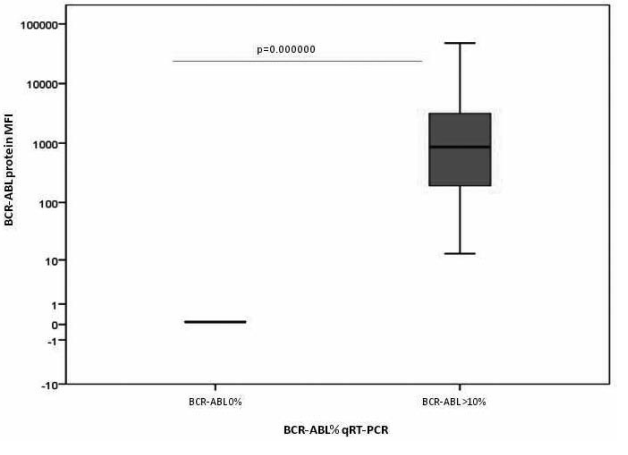

Figure 1.

Results. The BCR-ABL protein kit has the capability to produce a result in 4 hours. All CML patients were positive using the BCR-ABL protein kit. Conversely, all samples that were BCR-ABL1 negative by PCR showed no BCR-ABL1 protein. Samples from 22 healthy controls were negative by both methods. Where positive, BCR-ABL1 protein was readily detected in both total leukocytes and mononuclear cell (MNC) samples. Results were higher in MNC (P=0.014) likely due to absence of granulocytic enzymes. For CML patients with a BCR-ABL1 ratio >10%, the detection of BCR-ABL protein was highly significant

(P<0.001). For partially treated CML patients with a BCR-ABL1 ratio between 1-10%, the protein level was still significant, but treated CML patients with a BCR-ABL1 ratio <1% often had undetectable BCR-ABL1 protein. Using BCR-ABL1 positive K562 cells serially diluted with a BCR-ABL1 negative cell line (U937), the limit of sensitivity of the BCR-ABL1 protein assay was 0.4%. K562 cells cultured with 5 μM imatinib or 150 nM dasatinib for up to 96 hours showed a time dependent decrease in BCR-ABL1 protein level. *Summary/Conclusions.* No difference in results was observed when using PCR or the BCR-ABL1 protein kit. The BCR-ABL protein kit showed 100% accuracy with no false negative or positive findings, confirming that it can be used as an effective screening test for CML patient identification. Moreover, the BCR-ABL protein kit has the advantage that it can be rapidly (4 hours) performed in any laboratory with flow cytometry capabilities, eliminating the need for more elaborate time-consuming and costly techniques.

0228

N-RAS GENE MUTATIONS ARE INDICATIVE OF AN UNFAVORABLE OVERALL SURVIVAL PROGNOSIS IN PATIENTS WITH MYELODYSPLASTIC SYNDROMES, BUT NOT IN PATIENTS WITH ACUTE MYELOID LEUKEMIA

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Background. N-ras gene encodes small protein with GTPase activity, which is involved in the cell signal transduction. Point mutations alter the conformation of the protein resulting in its stable activation. Activating mutational changes of the N-ras gene are thought to play an important role in the development of numerous human cancers, including hematological malignancies. *Aims.* Point mutations of the N-ras gene were studied in patients with myelodysplastic syndromes (MDS) and *de novo* acute myeloid leukemia (AML) with the aim to evaluate its association with clinical outcome and disease progression. *Methods.* Using polymerase chain reaction and bidirectional direct sequencing, we analyzed 107 MDS patients (17xRA/RARS, 34xRCMD, 4x5q-, 23xRAEB1/2, 19xsAML, 10xMDS/MPS) and 73 *de novo* AML patients (5xM0, 14xM1, 21xM2, 6xM3, 21xM4, 5xM5, 1xM6) for point mutations in codons 12 and 13 of the N-ras proto-oncogene. *Results.* Mutations were present in 8% (9/107) of MDS patients and in 18% (13/73) of *de novo* AML patients. In MDS group, mutations were found mainly in codon 12 (7 of 9 mutations), two patients had mutations in both codons. Mutations were found with significantly higher frequency in patients with advanced MDS forms (8 of 9 mutations, P=0.014) in comparison to early forms. Significantly increased frequency of mutations in comparison to MDS was detected in AML (P=0.02). In AML patients, mutations were detected in codons 12 and 13 with an approximately same rate (7/13 and 6/13). Between AML subtypes, significantly increased frequency of the N-ras mutations was found in AML M2 subtype (6/13, P=0.018). All detected N-ras mutations lead to an exchange of amino acids. The most common observed base substitution was GGT to GAT in codon 12. In MDS patients we observed higher mutational variability, than in AML. MDS patients with N-ras mutations had significantly shorter survival period (median 9 months) than those without mutations (median 59 months, P=0.001). No difference in survival was obtained in AML patients with and without mutations (median 16 months, P=0.82). The presence of N-ras gene mutations was not associated with blasts count in analyzes tissue not in MDS, nor in AML patients. *Summary*. We showed that N-ras mutations are significantly associated with shorter overall survival period in patients with MDS, but not in patients with AML, although in this group were mutations detected with higher frequency. We proved the tendency of increasing number of detected mutations with disease progression towards advanced MDS forms. Thus, our findings point out the significance of the N-ras gene mutations in assessment of an individual MDS patients risk and usefulness as possible prognostic marker of MDS.

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Developmental biology and genomics

0229

BONE MARROW HAEMATOPOIESIS IN THE SECOND TRIMESTER OF FETAL LIFE IS STRONGLY BIASED TOWARDS B-LYMPHOPOIESIS AND IS SEVERELY IMPAIRED AT THE EBP-CBP TRANSITION IN DOWN SYNDROME

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Background. Children with Down syndrome (DS;trisomy 21;T21) have increased susceptibility to autoimmune disease and lymphopenia-associated infection as well as a 12-fold increased risk of B-acute lymphoblastic leukaemia (ALL), while T-ALL is rare, suggesting that T21 may specifically perturb B-lineage development. While acute myeloid leukaemia in DS children is characterised by an identifiable 'pre-leukaemic' phase with prenatal acquisition of GATA1 mutations which is preceded by expansion of fetal liver megakaryocyte-erythroid progenitors, DS-ALL does not present neonatally and a similar 'pre-leukaemic' phase has not been identified. Our lab recently showed that T21 itself causes increased self-renewal of CMP and strongly biases their differentiation towards the megakaryocyte-erythroid lineage. *Aims.* To test the hypothesis that T21 perturbs B-lymphopoiesis. *Methods.* We analysed B-lymphoid progenitors in fetal liver, bone marrow (BM) and cord blood (CB) in DS compared to disomic controls. *Results.* B-cell progenitors (BCP) at all differentiation stages were detectable in normal second trimester fetal liver where they formed 16.6±1% of the total $CD34^+$ population. The majority of normal fetal liver BCP were committed BCP ($CD34^{int}$-lo$CD19^+$;10.6±1.6% of $CD34^+$ cells) with a lower frequency of early B progenitors (EBP;$CD34^{hi}CD127^+CD19^{neg}CD10^{neg}$;5.1±1.1% of $CD34^+$). By contrast, in DS fetal liver, BCP frequency and numbers were severely reduced (4.0±1.3% of $CD34^+$) with particular reduction in committed BCP ($CD34^{int\text{-}lo}CD19^+$;0.7±0.25%) and relative preservation of EBP (2.4±1.1%) which was recapitulated by *in vitro* differentiation on OP9-stroma. By comparison with normal fetal liver, BCP were present in large numbers in normal second trimester fetal BM where they formed 51.1±3.4% of $CD34^+$ cells. Although all stages of BCP were present in second trimester BM, EBP formed 4.9±1% of $CD34^+$ and most BCP (45.1±4%) were committed CD19+BCP. Similar to DS fetal liver, BCP in second trimester DS BM were markedly reduced in numbers and frequency (21.2±3.2% of $CD34^+$ cells) predominantly due to low numbers of committed $CD19^+$BCP (14.5±3.4%) with a smaller reduction in EBP (3.1±0.4 vs 4.9±1% of $CD34^+$). JAK2 R683 mutations were not detected in any samples. BCP were also reduced in CB from term DS pregnancies compared with normal disomic term CB (5.5±0.8 vs 8.4±0.4%) due to a profound reduction in committed CD19+BCP (0.9±0.3 vs 3.9±1.2% of $CD34^+$) and, in contrast to DS fetal liver and BM, EBP in DS CB were increased compared to normal term CB (2.1±0.3 vs 0.9±0.2% of $CD34^+$). Numbers of mature B cells in DS CB were normal (12.8±0.2 vs 13.8±0.5%) suggesting compensatory expansion from the reduced BCP pool. *Conclusions.* These data show for the first time that: 1.all stages of BCP development occur in second trimester human fetal liver as well as BM; 2.B-lymphopoiesis is extremely active, constituting >50% of all haematopoiesis at the progenitor level, in second trimester BM; and 3.abnormal B-cell development in DS begins in fetal life with progressive block in EBP maturation to $CD19^+$ committed BCP. These abnormalities in DS are apparent in BM and CB, as well as fetal liver, suggesting they persist in post-natal life and may underlie the abnormal immune responses and increased susceptibility to B-ALL in children with DS.

0230

DEVELOPMENTAL STAGE-SPECIFIC INTERPLAY BETWEEN GATA1 AND IGF SIGNALING IN FETAL HEMATOPOIESIS AND LEUKEMOGENESIS

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Oncogene-mediated transformation of hematopoietic cells has been

extensively studied, but little is known about the molecular basis for restriction of oncogenes to certain target cells and differential cellular context-specific requirements for oncogenic transformation between infant and adult leukemias. Understanding cell type-specific interplay between signaling pathways and oncogenes is essential for developing targeted cancer therapies. Here, we addressed the vexing issue of how developmental restriction is achieved in Down syndrome acute megakaryoblastic leukemia (DS-AMKL), characterized by a triad of fetal origin, mutated GATA1 (GATA1s), and trisomy 21. We demonstrate overactivity of insulin-like growth factor (IGF) signaling in authentic human DS-AMKL and in a DS-AMKL mouse model generated through retroviral insertional mutagenesis. ShRNA mediated IGF1R knockdown in DS-AMKL cell lines (CMK, CMY) led to a dramatic reduction of transduced cells, whereas no substantial effect was observed in 'control' cell lines, including K562, CHRF288-11, and M-07. Pharmacological activation and repression of IGF1R signaling followed by *in vitro* culture, CFU assays and intracellular phosphoprotein staining showed that in both murine and primary human AMKL cells expressing GATA1s, the IGF/IGF1R/mTOR pathway is highly activated and is the major mitogenic pathway for growth. We observed a significant delay of leukemia onset upon IGF1R knockdown after transplantation of G1s-mAMKL cells into RAG2$^{-/-}$ recipients. Furthermore, we could show that fetal, but not adult, hematopoietic progenitors are dependent on IGF pathway. Gene set enrichment analysis revealed that the mitogenic activity of IGF1R is exerted at the transcriptional level by activation of E2F target genes. GATA1 restricts IGF-mediated activation of the E2F transcription network to coordinate proliferation and differentiation. Failure to directly interact and repress E2F transcription factors in mutated GATA1s converges with overactive IGF signaling to promote cellular transformation of DS fetal progenitors, whereas the hyperproliferative phenotype of Gata1s fetal MPs can be rescued by shRNA mediated repression of global E2F transcription activity. Thus, our study reveals a complex, fetal stage-specific regulatory network, underscores context-dependent requirements during oncogenesis and explains resistance to transformation of ostensibly similar adult progenitors.

0231

CPG-ODN 2006 AND HUMAN PARVOVIRUS B19 GENOME CONSENSUS SEQUENCES SELECTIVELY INHIBIT GROWTH AND DEVELOPMENT OF ERYTHROID PROGENITOR CELLS

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Background. Recent studies have shown that anemia is commonly observed following exposure to pathogens or pathogen-derived products, which are recognized via toll-like receptor (TLR) 9. TLR9 has evolved to recognize unmethylated CpG dinucleotides that are relatively common in bacterial and viral genomic DNA, but not in vertebral genomes. CpG DNA is generally most active as synthetic single-stranded (ss) oligodeoxynucleotide (ODN) sequences 20-30 nucleotides long, containing two to three CpG motifs with a modified nuclease-resistant backbone, typically a phosphorothioate (PS) backbone in which one of the non-bridging oxygen atoms at each of the natural (wild) phosphodiester (PO) linkages is replaced with a sulfur. Previous studies in mice have linked anemia and suppressed erythropoiesis induced by the CpG-ODN with PS backbone to indirect effects of proinflammatory cytokines produced by accessory cells. Of note is that CpG ODN with different backbones and different sequence motifs can induce dramatically different profiles and kinetics of immune activation. *Aims.* We aimed to precisely characterize the role of the best-characterized ligand for TLR9, CpG-ODN 2006, during hematopoiesis focusing on different backbones. *Methods.* Highly purified human CD34$^+$ cells were induced for proliferation and differentiation to erythroid lineage in the presence of interleukin-3, stem cell factor and erythropoietin (EPO) and the direct effects of CpG-ODN 2006 on hematopoietic progenitors were investigated focusing on different backbones. *Results.* CpG oligodeoxynucleotide (ODN)-2006 with PO (2006-PO) but not with the PS backbone, selectively inhibited the erythroid growth derived from human CD34$^+$ cells. The inhibitory effects of 2006-PO on erythroid growth depended on the ODN sequence and backbone, but not on the CpG-motif. 2006-PO was internalized by the erythroid progenitors within 30 min; however, expression of TLR9 mRNA was not detected in these cells. 2006-PO directly inhibited BFU-E growth, resulted in the accumulation of cells in S and G2/M phases, and increased cell-size and frequency of apoptotic cells. These features were similar to those observed in erythroid progenitors infected with human parvovirus B19 (B19) that causes pure red cell aplasia. The consensus sequence of 2006-PO was defined as 5'-GTTTTGT-3' which was located in the P6-promoter region of B19, and inhibited erythroid growth in a sequence specific manner and down-regulated expression of EPOR. 2006-PO down-regulated expression of erythropoietin receptor (EPOR) mRNA and EPOR, while did not affect the expression of GATA-2, GATA-1, friend of GATA-1 (FOG-1), EKLF and RPS19 mRNA. 125I-EPO binding assay showed a decrease of the specific binding of 125I-EPO to the cells treated with 2006-PO. B19 genome extracted from serum also inhibited erythroid growth and down-regulated expression of EPOR on glycophorin A+ cells. *Summary/Conclusions.* The data presented in the current study describe for the first time the presence of a ssDNA consensus sequence in both synthetic ODN-2006 and the B19 genome, and that this site selectively inhibits erythroid growth. The inhibition of erythroid growth by the consensus sequence was also accompanied by the inhibition of EPOR expression. These results provide a possible insight into our understanding of the mechanisms of human parvovirus B19-mediated inhibition of erythropoiesis.

0232

ESSENTIAL ROLE OF AN ANTI-APOPTOTIC MOLECULE ANAMORSIN FOR BOTH INTRINSIC AND EXTRINSIC REGULATION OF MURINE FETAL LIVER HEMATOPOIESIS

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Background and *Aims.* Anamorsin (AM, also called CIAPIN-1) is an anti-apoptic factor, which we originally isolated as a molecule that confers factor-independent survival on hematopoietic cells (J.Exp.Med. 199(4), 2004). AM has no structural homology to any known anti-apoptosis molecules such as Bcl-2 and IAP family members. AM-deficient (AM-/-) mice are embryonic lethal at late gestation due to the defect of definitive hematopoiesis. In this study, we performed a series of experiments to characterize the hematopoietic defects in AM-/- fetal liver (FL). *Methods.* The expression levels of AM in murine hematopoietic cells were evaluated by quantitative PCR (qPCR) method. FL hematopoietic stem cells (Lin-Sca1+c-Kit+, LSKs) were sorted from embryonic day (E) 14.5 wild-type (WT) and AM-/- FL and subjected to clonogenic assay, competitive repopulation (CRU) assay, and limiting dilution assay. LSKs isolated from E14.5 FL were cocultured with subconfluent FL stromal cells. After 7-days of incubation, hematopoietic cells floating from the stromal layer were collected and analyzed for colony forming activities. *Results.* AM was ubiquitously expressed in hematopoietic cells derived from E9.5-10.5 aorta-gonad-mesonephros (AGM) region, E14.5 FL, and adult bone marrow. qPCR analysis using E14.5 FL cells demonstrated that AM was more abundantly expressed in immature stem/progenitor cells than in mature cells expressing lineage-specific marker such as Mac1 or Ter119. Total number of hematopoietic cells was significantly decreased in all lineages in AM-/- FL compared to WT FL, which was more apparent in hematopoietic stem (% of LSK: 1.8 vs. 0.6 (n=5, P<0.05)) and progenitor (% of Lin-c-Kit+: 15.7 vs. 8.4 (n=5, P<0.05)) fractions. Also, *in vitro* colony assay, FL cells from E14.5 AM-/- embryos gave rise to less (approximately one third compared to WT) colonies in all colony types. In primary transplantation, there was a 5- to 8-fold reduction of donor-derived cells in each lineage in mice transplanted with AM-/- FL LSKs 4 weeks after transplantation. The limiting dilution assay showed that CRU frequency was severely decreased in AM-/- FL LSKs (1/755 in AM-/- vs. 1/177 in WT (n=18, P<0.05)). In addition, AM-/- FL LSKs had less long-term reconstituting ability in the secondary transplantation. Next, we analyzed whether AM deficiency might affect the function of FL stoma cells to support hematopoiesis. Interestingly, although stromal cells from WT FL supported colony-forming activities of AM-/- FL LSKs, those from AM-/- FL hardly supported growth of WT FL LSKs, indicating that, in addition to the defect in hematopoietic cells, the stromal function to support hematopoiesis was also impaired in AM-/- embryos. *Conclusions.* In AM-/- fetal liver, total number of hematopoietic cells was decreased, which was more prominent in immature hematopoietic stem/progenitor cells than in matured cells. Also, AM-/- hematopoietic stem/progenitor cells had less reconstituting activities in terms of the frequency of CRU and long-term maintenance. Further-

more, AM was required for the function of fetal liver cells to support hematopoiesis. These results indicate that AM deficiency causes both cell autonomous and extrinsic defects in fetal liver hematopoiesis. Further precise molecular mechanisms responsible for these defects will be presented in the meeting.

0233

TGF-BETA TRANSDUCED MESENCHYMAL STEM CELLS AMELIORATE AUTOIMMUNE COLLAGEN-INDUCED ARTHRITIS BY REGULATING TREG-TH17 CELLS AND OSTEOCLAST DIFFERENTIATION

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Bone marrow derived mesenchymal stem cells (MSCs) are multipotent cells that regulate immune responses. MSCs have recently been shown to prevent autoimmunity such as autoimmune encephalomyelitis (EAE) and diabetes. However, their therapeutic effect in rheumatoid arthritis (RA) remains controversial. In this study, we examined the therapeutic potential of TGF-β transduced MSCs in experimental autoimmune rheumatoid arthritis (RA), an accepted animal model of collagen-induced arthritis (CIA). We show that the systemic infusion of syngeneic TGF-β transduced MSCs prevented arthritis development and reduced bone erosion/cartilage destruction. Treatment with genetically modified MSCs expressing TGF-β potently suppressed type II collagen (CII) specific T cell proliferation and downregulated pro-inflammatory cytokine production. These therapeutic effects were associated with an increase in type-II collagen-specific $CD4^+Foxp3^+$ regulatory T cells and an inhibition of Th17 cell formation in the peritoneal cavity and spleen. Furthermore, TGF-β-MSCs migrated to inflamed joints, and such migrations inhibited osteoclast differentiation. Our results demonstrated that gene transfer of TGF-β to MSCs limits the development of arthritis in a model of CIA and joint inflammation. These data suggest that modulating T cell-mediated immunity by gene-modified MSCs provides a novel therapeutic approach for RA

0234

IDENTIFICATION OF LONG-TERM REPOPULATING HEMATOPOIETIC STEM CELLS WITHOUT NEGATIVE SELECTION

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Background. In the last two decades, identification of hematopoietic stem cells (HSCs) has been improved by using both multi-color FACS and *in vivo* competitive reconstitution assay. However, conventional staining protocols need many antibodies that include both positive and negative markers. Furthermore, information for location of HSCs in hematopoietic tissues is also important to understand HSC behavior and HSC-microenvironment (niche) interaction *in vivo*. Current methods for purification of HSCs largely depend on negative-selection such as lineage markers, CD34, Flk2 and CD48. Though negatively stained cells can easily be discriminated by FACS, identification of negative cells is very difficult by nonquantitative immunofluorescence on fixed and embedded tissue sections. Thus, simple method using only positive markers is preferable for fulfilling the above requirements. *Design and Methods.* In this study, we performed a stringent comparative gene profiling analysis to identify genes preferentially expressed in the HSC population. Among the selected HSC-specific genes, we focused on genes encoding cell surface protein, and identified plexin domain containing 2 (Plxdc2) as a novel positive marker for long-term HSCs. Plxdc2 is a single-pass type I membrane protein which is expressed in tumor vessels but not in most normal tissues. Because anti-Plxdc2 antibody is not available for FACS, we used Plxdc2::GFP knock-in mouse for subsequent analyses. *Results.* In whole bone marrow (WBM) of Plxdc2::GFP knock-in mouse, GFP^+ cells are less than 0.2%. Competitive reconstitution assay clearly showed that all HSCs are included in Plxdc2::GFP^+ fraction. Limiting dilution competitive repopulation assays revealed that one out of every 3.5±0.6 (29%) intravenously injected $Plxdc2^+$ $c\text{-}Kit^+$ $Sca\text{-}1^+$ Lineage cells long-term multilineage reconstituted irradiated mice. However, in WBM GFP^+ population, more than 50% of the cells are c-Kit-/low, $Sca\text{-}1^+$, Lineage- uncharacterized fraction. In order to further purify long-term HSCs in Plxdc2::GFP^+ cells, we investigated the additional marker. Throughout the screening of various known HSC-related markers, we selected CD150 (Slamf1). The frequency of $Plxdc2^+$ $CD150^+$ cells in WBM is only 0.01%, suggesting that long-term HSCs are highly enriched by only two positive markers. *Conclusions.* We propose new and simple method for identification of long-term HSCs without using negative markers. Single cell reconstitution assay of $Plxdc2^+$ $CD150^+$ cells, immunofluorescence analysis on bone sections to detect HSCs, production of anti-Plxdc2 monoclonal antibody and analysis of Plxdc2-deficient mice (Plxdc2GFP/GFP mice) are our ongoing tasks.

0235

THE ASB2α UBIQUITIN LIGASE COMPLEX AS A REGULATOR OF THE CROSSTALK BETWEEN HEMATOPOIETIC STEM CELLS AND THEIR MICROENVIRONMENT

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Background. Understanding the molecular mechanisms controlling early steps of hematopoietic differentiation that are deregulated in leukemia cells and characterizations of properties of hematopoietic stem cells (HSCs) and leukemic stem cells are major challenges not only for the development of therapeutic approaches to targets leukemic stem cells *in vivo* but also for engraftment of normal HSCs following transplantation. Our previous work identified the *ASB2* gene as a retinoic acid response gene and as a target gene of the oncogenic PML-RARα fusion protein in acute promyelocytic leukemia cells. However it is specifically expressed in normal immature hematopoietic cells and so is likely to be relevant during early hematopoiesis. The *ASB2* gene encodes ASB2α protein that is the specificity subunit of an E3 ubiquitin ligase complex and that triggers polyubiquitination of Filamins leading to their degradation by the proteasome. We recently demonstrated that ASB2α, through Filamin degradation, can regulate integrin-dependent functions. Because integrins play a major role in HSC adhesion to the hematopoietic niche, ASB2α may modulate the balance between adhesion and migration of HSCs and therefore their cell fate within the hematopoietic niche. *Aims.* Functional assays and molecular approaches were performed to get structural and functional insights into the ASB2α E3 ubiquitin ligase complex. *Methods and Results.* To assess the role of ASB2α in hematopoietic cell adhesion, hematopoietic cells stably transfected with inducible vectors encoding ASB2α or an E3 ubiquitin ligase defective mutant were labeled and allowed to adhere on wells coated with fibronectin, the main ligand of β1 integrins. We show that ASB2α enhances adhesion of hematopoietic cells to fibronectin. Furthermore, this effect was recapitulated in Filamin knockdown cells. To further investigate ASB2α function, structural and cell biology studies were carried out. By structural homologies and site-directed mutagenesis, we have defined regions of ASB2α and Filamin proteins that are necessary for co-localization and subsequent ASB2α-induced Filamin degradation. Importantly, the defined region of ASB2α involved in this process is similar to the binding domains of other Filamin ligands involved in cell motility. Thus, ASB2α may displace Filamin from these proteins before triggering Filamin polyubiquitylation and degradation by the proteasome. *Summary/Conclusions.* Our results demonstrated that ASB2α is a regulator of integrin-dependent adhesion and initiation of migration of hematopoietic cells. This control of hematopoietic cell motility derives from a specific determinant of ASB2α that is involved in the recruitment of its substrate (Filamin) leading to its subsequent degradation. The hallmark of HSCs is their repopulation and motility (adhesion and migration) potentials. Indeed, HSC mobilization, homing, and repopulation are sequential events with physiological roles. Because interactions between HSCs and their microenvironment are broken during cell mobilization and re-established to enable efficient bone marrow homing and retention, ASB2α may represent a novel pathway controlling the status of HSCs within the hematopoietic niche and thus, a potential drug target for therapeutic strategies involving HSC engraftment.

0236

TNF-ALPHA UPREGULATES THE EXPRESSION OF HES1, HEY1 AND GATA3 IN HEMATOPOIETIC STEM CELLS, IN A NOTCH-DEPENDENT WAY, DURING T-LYMPHOPOIESIS

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Background. We have previously shown that umbilical cord blood (UCB) hematopoietic stem cells (HSC), as compared to bone marrow (BM) HSC, express higher levels of NOTCH and NF-κB pathway components and transcriptional targets. Those differences would relate to the higher potential to generate T-lymphocytes observed for UCB HSC, *in vitro* and *in vivo*. In line, treatment of HSC with TNF-α, an agonist of the NF-κB pathway (also expressed at higher levels in UCB HSC), increases the generation of T-cells. Nevertheless, the molecular mechanisms by which TNF-α promotes T-lymphopoiesis are not known. *Aims.* To evaluate the influence of TNF-α in Notch signaling. Material and *Methods.* Human CD34$^+$ HSC were immunomagnetically purified from UCB (obtained after informed consent) and co-cultured (12h) with cells expressing the Notch ligand Delta like 1 (OP9-DL1), or not (OP9), and in the presence or absence of TNF-α (0.25 ng/mL) and the Notch inhibitor DAPT (10 nM). The same culture conditions were carried with HSC pre-treated with the protein synthesis inhibitor cycloheximidine (CHX). Real-time PCR was used to quantify NOTCH transcriptional targets (GATA3, HES1 and HEY1). GAPDH levels were used for normalization and relative expression levels were obtained using HSC co-cultured with OP9 cells as reference samples. *Results.* Transcript levels of HEY1 and HES1 were upregulated upon co-culture with OP9-DL1 cells. Interestingly, their levels were further up-regulated by TNF-α, but, only in the absence of DAPT, indicating a Notch-dependent positive effect. As expected for direct Notch targets, the upregulation induced by OP9-DL1 cells occurred even in HSC treated by CHX, nevertheless, while CHX treatment further increased the expression of HES1, the induction of HEY1 was reduced. These latter results indicate that proteins synthesized during the co-culture period may act negatively in the transcription of HES1 and positively on HEY1. In line, the effect of TNF-α on HES1 expression was higher on CHX treated HSC, as compared to non treated cells, while, the effect of TNF-α on HEY1 expression was lower on CHX treated HSC. Despite these changes, the positive effect of TNF-α was still present in CHX treated HSC, indicating the existence of a molecular signaling mechanism independent of *de novo* protein synthesis. For GATA3, no upregulation was evident in HSC co-cultured solely with OP9-DL1 cells, nevertheless, CHX and TNF-α upregulated GATA3 expression additively. Finally, in the presence of both, CHX and TNF-α, DAPT reduced the expression of GATA3, indicating a dependence on Notch signaling. Summary. Our results indicate that the positive effect of TNF-α on T-lymphopoiesis may partially derive from a Notch-dependent molecular signaling mechanism (independent of *de novo* protein synthesis) that contributes for the transcriptional upregulation of Notch targets. Furthermore, our results indicate the existence of positive and negative feedback mechanisms, acting trough *de novo* synthesized proteins, controlling the expression of HEY1 and HES1, respectively.

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0237

ADIPOSE TISSUE AS A DEDICATED RESERVOIR OF FUNCTIONAL MAST CELLS

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Background. Adipose tissue (AT) has long been studied for its metabolic role. Since ten years, numerous studies have focused on the hematopoietic mature cells harboured in this tissue, i.e. lymphocytes and macrophages. These mature resident cells have been largely studied for their involvement in the onset of obesity. Beside mature macrophages and lymphocytes, the presence of hematopoietic stem cells (HSC) has been proposed although their differentiation potentials are still questioned. *Aims.* The aim of this study was to identify potential HSC in murine AT and to characterize their function both *in vitro* and *in vivo*. *Methods.* Putative murine adipose derived HSC were identified by flow cytometry by using cell surface antigens described in BM (KTLS phenotype). These cells were then sorted and used for both *in vitro* and *in vivo* studies. *in vitro*, KTLS cells were plated in long term cultures (LTC) and colony forming cell (CFC) assays. Colonies generated in both types of culture were phenotypically and functionally characterized. In parallel, competitive repopulation assays were performed with adipose derived KTLS cells, in order to quantify and analyze their progeny *in vivo*. *Results.* AT harbours numerous c-kit$^+$/CD90$^{lo/Lin-}$/Sca1$^+$ (KTLS) cells, with a frequency 100 times higher than in BM. *in vitro*, these KTLS cells generate both macrophages and mast cells in LTC, and differentiate only into functional mast cells in CFC assays. After *in vivo* transplantation, adipose-derived KTLS preferentially migrate towards organs such as AT, intestine, lung and skin where they generate mast cells and macrophages but failed to commit to lymphoid lineage. *Summary/Conclusions.* Our results demonstrate that AT is a source of KTLS cells dedicated to generate mast cells and macrophages. Due to its abundance in adult organism, AT would thus constitute a major source of myeloid progenitors able to home to different organs. The identification of such cells in human AT would be of interest in cell therapy.

0238

PARTICIPATION OF THE PROGENY OF MARKED BY LENTIVIRAL VECTOR MESENCHYMAL STEM CELLS IN THE DEVELOPMENT OF HEMATOPOIETIC MICROENVIRONMENT

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Background. Colony forming units fibroblast (CFU-F) are the polypotent progeny of mesenchymal stem cells (MSCs). Role of CFU-F in the formation of all elements of hematopoietic stromal microenvironment is still obscure. Proliferative potential of CFU-F is limited and unlike MSCs they are not capable of transfering hematopoietic microenvironment in mice. After implantation of femur bone marrow plug or adherent cell layer (ACL) from long-term bone marrow culture (LTBMC), but not CFU-F derived colonies, under renal capsule of syngeneic recipients the ectopic hematopoietic foci form. Stromal cells in such foci are derived from donor MSCs while hematopoietic cells have recipient's origin. The size of the foci formed is proportional to the femur equivalent transplanted and can be used for semi-quantitative determination of MSC number. *Aims.* The aim of the study was to investigate the involvement of marked progeny of murine MSCs in formation of stromal hematopoietic microenvironment. *Methods.* ACLs of 2-week-old LTBMCs were infected with 100 mkl of concentrated (108 viral particles/mL) self-inactivating (SIN) HIV LeGO vector encoding EGFP (all plasmids and Phoenix cells were placed by Prof. Boris Fehse) in 3 ml of alfaMEM with 10% FCS and 8 mkg/mL polybrene for 6 hours. In 2, 8, 14 and 16 weeks after the infection ACL cells were trypsinized and plated 25, 50, 100 and 200 cells/well of 96-well plate for CFU-F analysis in alfaMEM supplemented with 20% FCS and 5 ng/mL FGF2. In separate experiments 2-3 weeks after infection ACLs were implanted under the renal capsule of syngeneic mice. In 6 weeks the number of marked CFU-Fs was measured among nucleated cells from the foci formed. *Results.* In 2, 8, 14 and 16 weeks after infection the proportions of marked cells in infected ACLs were 9.7, 31, 5 and 8.8% correspondingly. The efficiency of marked CFU-Fs cloning was 1 cell per 118±39 on week 8 and per 690±280 on week 14. Obviously the number of marked stromal cells in the ACLs is proportional to the number of marked CFU-F.ACLs implanted under the renal capsule of syngeneic mice contained 19.5±8.0% EGFP positive cells. The size of the foci formed was (3.3±0.5)×10^6 cells. Most of the tiny bones developed in the foci were EGFP positive as revealed PCR analysis. The concentration of CFU-Fs in ectopic foci formed was 33.5±21.7 per 106 cells that is similar to the concentration of CFU-Fs in the bone marrow. The proportion of marked CFU-Fs among total CFU-Fs was 8.97±1.18%. *Conclusions.* Revealed interconnection between the frequency of marked CFU-Fs and the proportion of marked differentiated stromal cells in the ACLs suggests that exactly CFU-Fs are the progenitors of developed hematopoietic microenvironment. Marked with lentiviral vectors MSCs are able to transfer hematopoietic microenvironment at least twice and keep the ability to produce the considerable number of marked progeny. The data indicate the advantages of MSCs as targets for gene therapy due to their longevity, ability to self-renew and to produce the differentiated progeny with the integrated gene of interest.

0239

VITAMIN D RECEPTOR (VDR) INDUCTION IS CRITICAL FOR LANGERHANS-TYPE DC LINEAGE COMMITMENT OF COMMON HUMAN MONOCYTE/DC PROGENITORS

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Langerhans cells (LCs) represent highly abundant dendritic cells (DCs) that form tight networks of hematopoietic cells in stratified epithelial tissues, however lineage commitment processes leading to their differentiation from hematopoietic stem cells remain poorly defined. The cytokine TGF-β1 is both sufficient and necessary for the induction of LC generation from human myeloid progenitor cells *in vitro*, and is similarly required for murine LC differentiation *in vivo*. We here searched for transcription factors induced or repressed by TGF-β1 concomitant with LC lineage commitment of myeloid progenitor cells. $CD34^+CD45RA^+CD19^-$ umbilical cord blood cells were stimulated for 48 h *in vitro* using a cytokine combination that induces common monocyte/LC progenitor cells. TGF-β1 addition to these cells induces LC commitment, while in its absence, cells alternatively differentiated into monocytes. Gene array profiling identified the VDR to be rapidly induced (within 6 h) during TGF-β1-dependent LC commitment. Ectopic retroviral VDR expression strongly augmented LC induction, whereas VDR inhibition abrogated LC differentiation. In line with this, epidermal LCs show a nuclear VDR expression pattern. Therefore, VDR induction downstream of TGF-β1 promotes LC commitment of shared monocyte/LC progenitors.

0240

HDAC AND SIRT INHIBITION DIFFERENTIALLY MODULATE CELL FATE DECISIONS DURING NEUTROPHIL AND MEGAKARYOCYTE DIFFERENTIATION

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Background. The clinical use of chromatin modulating drugs, such as histone deacetylase (HDAC) inhibitors, for the treatment of bone marrow failure and hematopoietic malignancies has increased dramatically over the last few years. In addition, the use of a distinct group of HDAC inhibitors, SIRT-inhibitors, is being investigated in pre-clinical studies. Nonetheless, little is currently known concerning the effects of both class I/II HDAC- and SIRT-inhibitors on normal myelopoiesis. *Aims.* To compare and contrast the effects of HDAC inhibitors on normal myeloid development and identify specific targets of HDAC inhibition in hematopoietic progenitors *Methods*. We utilized an ex-vivo differentiation system in which umbilical cord blood derived $CD34^+$ cells were treated with trichostatin A (TSA), sodium butyrate (SB) and valproic acid (VPA) to evaluate the effect of HDAC inhibitor treatment on myeloid lineage development, proliferation, and terminal neutrophil differentiation. In addition we investigated the effects of VPA and the SIRT-inhibitor nicotinamide (NAM) on megakaryocyte/erythroid progenitor (MEP) differentiation and terminal megakaryocyte differentiation. *Results.* TSA treatment modestly reduced progenitor proliferation, while SB and VPA resulted in concentration dependent effects on proliferation and apoptosis. Addition of VPA uniquely stimulated $CD34^+$ proliferation, while SB treatment both quantitatively and qualitatively inhibited terminal neutrophil differentiation. Addition of 100 μM VPA resulted in increased numbers of mature neutrophils with a block in differentiation at increasing concentrations. VPA treatment increased the percentage and absolute number of MEPs, yet resulted in a dramatic decrease in terminal megakaryocyte differentiation. In contrast with the effects of VPA on megakaryocyte development, addition of the SIRT inhibitor NAM resulted in a significant increase in the percentage of mature megakaryocytes and increase in polyploidy. However, this effect was accompanied by a significant decrease in megakrayocyte progenitor proliferation. Preliminary results have demonstrated that treatment of $CD34^+$ progenitors with 100 μM VPA in combination with 1mM NAM inreased both the precentage and absolute number of mature megakaryocytes. Treatment of myeloid progenitors with all HDAC inhibitors resulted in increased histone acetylation and TSA, SB and VPA were found to also have differential effects on the acetylation of non-histone proteins. *Summary/Conclusions.* Individual HDAC inihibitor treatment has specific effects on cell fate decisions during myeloid development. These data provide novel insights in the effects of HDAC inhibitors on regulation of normal hematopoiesis and are of importance when considering utilizing these compounds for the treatment of myeloid malignancies and bone marrow failure syndromes. Moreover, these data confirm the distinct roles of class I and II HDACs (inhibited by TSA, SB and VPA) compared to class III HDACs, better known as Sirtuins (inhibited by NAM) in normal hematopoiesis. Together, these data underline the important role of acetylation in regulation of normal hematopoiesis and thereby contribute to a better understanding of the pathogenesis of myeloid malignancies

0241

PHENOTYPIC AND FUNCTIONAL CHARACTERISTICS OF TISSUE NON SPECIFIC ALKALINE PHOSPHATASE (TNSALP) POSITIVE BONE MARROW FIBROBLASTIC RETICULAR CELLS IN MURINE RADIO-INDUCED APLASIA

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Background. Bone marrow aplasias are characterized by a peripheral blood cells deficit resulting from a deficiency in the production of one or several blood cells lineages in bone marrow. Some of these aplasias are able to regenerate, others don't respond to treatment. In most of the cases, spontaneously regenerating bone marrows are characterized by the presence in the whole marrow cavity, of fibroblastic cells presenting a strong alkaline phosphatase activity (ALP), this activity has completely disappeared in irreversible bone marrow aplasias. *Aims.* The aim of our work is to analyze phenotypic and functional characteristics of these ALP positive reticular cells and to test a possible role of ALP in haematopoietic regeneration. *Methods.* Mice were submitted to a total body irradiation with a 4Gy rate of gamma rays. Femoral bone marrows were removed at several time points after irradiation and put in culture in adequate conditions to form CFU-Fs colonies. CFU-Fs from non irradiated mice were used as control. Phenotype of CFU-Fs was analysed, and their haematopoietic supportive capacities were tested. *Results.* In a first step, we have studied ALP positive cells and haematopoietic cells evolution in a murine model of reversible radio-induced aplasia. We have demonstrated that the density of the ALP network and the number of ALP positive cells increase strongly in the first three days following irradiation. This increase precede haematopoietic regeneration and isn't a result of ALP positive cells proliferation. *in vitro* studies have also demonstrated that this increase in ALP activity is due to the expression of both TNSALP (tissue non specific alkaline phosphatase) and PMCA whose mRNA expression progressively increase after irradiation. Observations suggest that ALP cells differentiated from mesenchymal stem cells: the number of CFU-Fs is proportional to the number of *in situ* bone marrow ALP cells and the proportion of ALP positive cells increase in CFU-Fs from irradiated bone marrows, ALP cells keep self renewal and differentiation capacities and express common marker of mesenchymal stem cells ($CD106^+$, $Sca\text{-}1^+$, $CD11b^-$, $CD45^-$ and $flk1^-$). They also expressed pre-osteoblastic markers (runx2 and osteocalcin). To test their function in haematopoiesis, we have realized co-cultures of CFU-Fs and haematopoïetic precursors ($ckit^+$, $sca\text{-}1^+$, lin^-). We observed that, CFU-Fs from irradiated bone marrow, characterized by a high proportion of TNSALP positive cells and a strong expression of sdf-1 (stroma derived factor-1), strongly stimulate the proliferation of haematopoietic precursors and induce their differentiation into the myelo-monocytic and granulopoietic lineages. Studies at a late embryonic stage, on TNSALP deficient mice demonstrated a deficit in granulopoiesis as well as in the growth of the myelo-monocytic lineages. *Summary/conclusions.* All these data suggest that TNSALP reticular cells, directly derived from mesenchymal stem cells play a role in normal haematopoiesis and in regeneration of bone marrow aplasia by inducing proliferation and differentiation of myelo-monocytic and granulocytic precursors by a mechanism still to be elucidated.

0242

IFNα ACTIVATES DORMANT HSCS *IN VIVO*

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Haematopoietic stem cells (HSCs) in mouse bone marrow are located in specialized niches as single cells. During homeostasis, signals from this environment keep some HSCs dormant, which preserves long-term

self-renewal potential, while other HSCs actively self renew to maintain haematopoiesis. In response to haematopoietic stress, dormant HSCs become activated and rapidly replenish the haematopoietic system. The signalling molecules promoting the exit of HSCs out of the dormant stage remain largely unknown. We have recently uncovered that in response to treatment of mice with interferon-alpha (IFNα), HSCs efficiently exit G0 and enter an active cell cycle. HSCs respond to IFNα treatment by increased phosphorylation of STAT1 and PKB/Akt, expression of IFNα target genes and up-regulation of stem cell antigen-1 (Sca-1). HSCs lacking either the interferon-α/β receptor (IFNAR), STAT1 or Sca-1 are insensitive to IFNα stimulation, demonstrating that STAT1 and Sca-1 mediate IFNα induced HSC proliferation. Since the induced activation of HSCs upon IFNα treatment is only observed *in vivo* and not *in vitro*, we are examining the change in HSC-niche interactions upon IFNα stimulation. Although dormant HSCs are resistant to the anti-proliferative chemotherapeutic agent 5-FU, HSCs pre-treated (primed) with IFNα and thus induced to proliferate are efficiently eliminated by 5-FU exposure *in vivo*. Conversely, HSCs chronically activated by IFNα are functionally compromised and are rapidly out competed by non-activatable IFNAR$^{-/-}$ cells in competitive repopulation assays. Data on a possible role for STAT1 and Sca-1 in this chronic IFNα treatment will be presented. In summary, while chronic activation of the IFNα pathway in HSCs impairs their function, acute IFNα treatment promotes the proliferation of dormant HSCs *in vivo*. These data may help to clarify the so far unexplained clinical effects of IFNα on leukemic cells, and raise the possibility for novel applications of type I interferons to target cancer stem cells.

0243

BONE MARROW STROMA FROM MULTIPLE MYELOMA PATIENTS EXHIBITS MODIFIED RESPONSES TO TOLL-LIKE RECEPTOR ACTIVATION

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In human multiple myeloma (MM) the tumor cells exhibit strict dependence on bone marrow (BM) stromal elements. It has been suggested that in turn, MM cells modify multipotent stromal cells (MSCs) of the BM, and thereby the functions of MSCs may be compromised. We did not find phenotypic differences between MSCs from the bone marrow of MM patients and control donors. However, the analysis of responses of MSCs to toll-like receptor (TLR) ligands, revealed intrinsic differences between the MM and control populations. The activation of TLR-2 and TLR-4 by corresponding ligands augmented MSC proliferation in both MM and control MSCs. Different TLR ligands induced divergent effects on MSCs stimulated into adipogenesis and osteogenesis, ranging from augmentation to inhibition of differentiation. MM and control MSCs responded by IL-6, IL-8 and activin A secretion to TLR activation. However, in MSC derived from MM patients IL-8 secretion and ERK1/2 phosphorylation diverged from that of controls in response to TLR-2 activation. The persistence of these changes in long-term passage MSCs from MM patients suggests that these cells are intrinsically modified and consequently respond differently to TLR activation.

0244

AMPK INHIBITION INDUCES APOPTOSIS IN PEDIATRIC B-ALL CELLS WITH MLL GENE REARRANGEMENTS

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Background and Aims. Remarkable progress has been made in the past decade in pediatric Acute Lymphoblastic Leukemia (ALL) treatment, reaching cure rates of about 80%, but therapy is not yet effective in all cases. Infants with MLL gene rearrangements form the most striking example of patients who have not benefited from the improved treatment regimens. Consequently, current interest focuses on identifying new specific molecular targets to find new patient-tailored therapies. Thus, to identify aberrantly activated signal transduction pathways in MLL-rearranged patients, we used Reverse Phase Protein Microarrays (RPMA). This innovative technique can be used to quantify a highly multiplexed "portrait"of hundreds of signalling proteins at once from small clinical samples in a very reproducible, precise, sensitive and high-throughput manner. We further investigate RPMA results with *in vitro* studies testing the effects of a specific kinase inhibitor on apoptosis induction in leukemia cell lines. *Methods.* We compared with RPMA the signal transduction pathways working state of 8 MLL-rearranged patients vs 41 without known genomic translocations ones. The informed consent was obtained from all patients following the tenets of the Declaration of Helsinki. Phosphorylation status of 92 signalling proteins was analyzed. Based on RPMA results, we tested through proliferation and apoptosis assays the effect of Compound C, an AMPK inhibitor, on selected B-ALL human cell lines: 2 MLL-rearranged (SEM and RS4;11) and 2 non-translocated (MHH-CALL-2 and MHH-CALL-4). We then performed additional experiments to describe Compound C-induced apoptosis. *Results.* MLL-rearranged patients show an hyper-activated pathway that, through AMPK phosphorylation, leads to BCL-2 activation. Selected cell lines respond very differently to AMPK inhibition. GI50 (Growth Inhibition) at 48h is 0.2 μM for SEM, 3 μM for RS4;11, and 26 μM for non-translocated cell lines. LC50 (Lethal Concentration) at 48h is 7.5 μM for SEM, 8.5 μM for RS4;11 and 38 μM for non-translocated cell lines. Compound C treatment induces activation of Caspase-3, mitochondrial depolarization, ROS production, PARP cleavage, and DNA fragmentation. *Conclusions.* Our results thus demonstrate that the AMPK pathway is hyperactivated in MLL-rearranged patients, and it appears to directly contribute to the survival of MLL-rearranged cells. This study emphasizes the importance of protein pathway analysis as a route for discovery of functional derangement that may be functional, causative agents of cancer. Our data suggest AMPK as a new molecular target and encourage further studies of AMPK inhibitors as potential new drugs for treatment of MLL-rearranged leukemia patients.

0245

INTEGRATION OF GLOBAL SNP-BASED MAPPING AND EXPRESSION ARRAYS WITH MICRORNA PATTERNS REVEALS DEREGULATED MIRNAS AND THEIR GENE CANDIDATE TARGETS IN ACUTE MYELOID LEUKEMIA

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Acute myeloid leukemia (AML) is a heterogeneous clonal disease that results from multiple genetic alterations in hematopoietic stem cells. Genetic aberrations have decisive importance in the biology and clinical outcome of patients with AML; however, most patients relapse within 1-2 years. Therefore, improved predictive biomarkers are needed to identify other genetic alterations that collaborate in AML development. Deregulated miRNA expression plays a crucial role in tumorigenesis. Recent studies have shown some of the mechanisms through miRNA deregulation contribute to cancer: mutations, chromosomal translocations, epigenetic alterations, or a defective miRNA biogenesis. However, the role of DNA copy number variations (CNV) in deregulation of miRNAs in AML remains unclear. Our aim was to identify if CNV affects the deregulation of miRNAs in AML, and if that could affect the expression levels of their target genes. We analyzed 16 AML cell lines using 500K-SNP arrays (aSNP) (Affymetrix), analysis of 250 miRNAs by q-RT-PCR, and mRNA expression arrays (HG-U133A). After analyzing CNV in the cell lines, we evaluated if a significant gene dosage effect could be identified in the expression levels of specific miRNAs located within CNV segments generated by the genome-wide analysis. We identified 17 miRNAs with a significative correlation ($P<0.05$) between their expression and the CNV of the region: 14 up-regulated and located in a genomic region of amplification, and 3 down-regulated miRNAs, localized in regions with genomic deletions. The 14 miRNAs up-regulated comprise 5 clusters in 2 different amplified regions. MiR-100 and miR-125b, clustered in 11q24.1, have been described to be highly expressed in pediatric AML. There were 4 miRNA clusters on 14q32.31. In cluster A are located miR-379 and miR-494 ($P<0.001$), that have been described to control neuronal development. Cluster B is integrated by miR-127, miR-432, and miR-433, expressed in a compact space by using overlapping coding regions, which are implicated in fetal development. Interestingly, miRNAs belonging to clusters C and D of this region have been described to be up-regulated in adults with AML, (miR-154, miR-409-5p, and miR-370, miR-376A, miR-382, miR-134, and miR-485-5p) ($P<0.001$). Only 3 down-regulat-

ed miRNAs were located in regions with deletions: miR-15a (P=0.001) and miR-16 (P=0.033), clustered in 13q14.3; and miR-7 (P=0.05), located on 9q21.32. The implication of these miRNAs in tumorigenesis is well known. These results prompted us to perform a correlation between these miRNAs and the mRNA arrays in the cell lines to assess if their predicted target genes are also affected. We obtained a set of genes candidates whose altered expression is likely consequence of an aberrant miRNA regulation. In conclusion, the deregulation of some miRNAs could be explained by their location in genomic regions affected by genomic aberrations. Moreover, our integrative approach by aSNP, mRNA arrays, together with miRNA expression patterns in AML cell lines allowed us to identify 17 miRNAs, which could have an important role in AML, and their putative targets. Further functional studies are in progress.

0246

HYPOXIA MAINTAINS STEMNESS OF ENDOTHELIAL PROGENITOR CELLS

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Vascular homeostasis, remodeling and regeneration are maintained by tightly regulated vessel wall-derived somatic endothelial colony-forming progenitor cells (ECFCs). Despite promising experimental data, both cellular therapeutic vasculogenesis as well as anti-angiogenic therapy targeting ECFCs are of rather limited efficiency in clinical trails. A better understanding of vascular biology is thus urgently needed to develop alternative concepts for vascular regeneration and anti-angiogenic cancer therapy. Currently we believe that hypoxia in ischemic and tumor tissue is a key factor driving the revascularization machinery. *in vivo*, most cells exist under a defined oxygen pressure considerably below air oxygen. *in vitro*, isolated cells are usually treated under air oxygen conditions and encounter sudden reduced oxygen when re-injected for therapy. We hypothesized that differences between ex vivo and *in vivo* oxygen levels influence ECFC function and thus represent a key factor in vascular regenerative therapy. NORMOXIA has been used variably in the literature describing a wide spectrum of oxygen levels. We therefore designated the oxygen level present *in vivo* in the venous environment to be termed EUOXIA (41.5±3.4 mmHg) throughout this study. Oxygen levels above EUOXIA are defined as HYPEROXIA (139.8±2.9 mmHg) and below EUOXIA as HYPOXIA (27.4±7.3 mmHg). Adult human ECFCs were isolated and propagated directly from whole venous blood using a novel recovery strategy. During cell culture, pooled human platelet lysate (pHPL) entirely replaced fetal bovine serum (FBS). Progenitor cell phenotype, hierarchy, long-term proliferation, wound repair as well as migratory and vasculogenic functions were monitored under Euoxia as compared to hypoxic conditions or air oxygen levels commonly used in standard laboratory practice. Molecular regulation of cellular responses to different oxygen levels was assessed by flow cytometry and proteomic profiling. ECFC colony size was decreased under hypoxic compared to euoxic oxygenation, paralleled by a loss of high proliferative potential (HPP) ECFCs under Hypoxia. The absolute colony number (3.2±0.2 colonies per ten ECFCs seeded per cm^2) remained unchanged independent of oxygen levels. Hyperoxic conditioning resulted in an increase in proliferation in primary and long-term cultures and significantly augmented HPP colonies (60±18% of total colonies) as compared to hypoxic (0%) and euoxic (9±6%) oxygen levels. Vascular wound repair in scratch assays and *in vitro* Matrigel® vascular-like network formation was improved with escalating oxygen supply. Reoxygenation of hypoxic and euoxic ECFCs even lead to enhanced proliferation and function. Proteomic analysis of ECFCs identified several heat shock and antioxidant proteins involved in the oxygen-dependent regulation of migratory and proliferative responses. Reversible inhibition of ECFC function by hypoxic and euoxic oxygen levels indicates the presence of a silencing mechanism. These data suggest that hypoxic and euoxic oxygen levels maintain the ECFC stem/progenitor cell characteristic. Culture conditions for the clinical scale expansion of ECFCs may play a decisive role for their efficiency in therapeutic vasculogenesis. Revascularization of hypoxic tissues after ischemic injury by ECFCs must be indirectly mediated and are therefore further investigated.

0247

GENOMIC STABILITY IN LONG-TERM CULTURED MESENCHYMAL STROMAL/STEM CELLS

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Human multipotent mesenchymal stromal cells (MSCs) are currently being tested in clinical trials for immunomodulatory and regenerative therapies. We and others have recently established MSC propagation with pooled human platelet lysate (pHPL) as a substitute for fetal bovine serum (FBS). As serious doubts regarding the use of MSCs cultured with FBS and their possible genomic instability have arisen, we investigated safety aspects of short- and long-term cultures with FBS in comparison to pHPL. Unmanipulated bone marrow aspirates were seeded in alpha-MEM with pHPL. Clinical-scale expanded MSCs were harvested directly after primary culture (short-term). Representative cultures were continued for long-term expansions where each directly compared pHPL and FBS stimulation for a maximum of 46 to 51 population doublings until MSC proliferation ceased. Comparative genome hybridization (array-CGH) was carried out with short- as well as long-term expanded MSCs using a whole genome microarray platform and CGH analysis software. pHPL is highly efficient in stimulating MSC expansion resulting in 780±150 million MSCs after one passage. Flow cytometry revealed more than 95% viability, more than 95% CD73/90/105 reactivity and less than 2% hematopoietic contamination. We were able to show adipo-/osteo-/chondrogenic differentiation potential, endotoxin levels below 0.025 EU/mL and negative bacterial/fungal/mycoplasma testing. In all short-term MSC products, array-CGH revealed balanced genomic profiles. We detected several small copy number variations previously found in healthy individuals not associated with phenotype changes. In contrast, after long-term culture with FBS as well as pHPL, MSCs showed *de novo* copy number amplifications not reported in the database of genomic variants. Despite a high proliferation rate in the short-term pHPL-driven cultures, MSCs showed genomic stability according to array-CGH *Results.* These data support our earlier findings that MSCs expanded under humanized conditions did not form tumors *in vivo* in animal experiments. It is not clear whether *in vitro* genomic variations in long-term propagated MSCs under humanized as well as xenogeneic culture conditions may be associated with the risk of malignant transformation rather than representing replicative senescence. Therefore safety concerns have to be vigilantly addressed parallel to the clinical use of MSCs.

0248

PHOSPHOPROTEOMIC PROFILING OF LEUKEMIA CELLS

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Protein phosphorylation on serine, threonine, and tyrosine is well established as a crucial regulatory posttranslational modification in eukaryotes. While conventional biochemical methods failed to obtain a snapshot of the phosphoproteomes, advances in mass spectrometry (MS) methods have paved the way for indepth mapping of phosphorylation sites. Recently, we developed a phosphopeptide enrichment method using hydroxy acid-modified metal oxide chromatography (HAMMOC) with titania and zirconia (Rappsilber J et al., Nat. Protoc 2007). In HAMMOC, thousands of phosphorylated sites can be identified from limited protein amounts (<100 microgram per sample), enabling the application to the clinical tissue samples. HAMMOC can work effectively for clarification of pathways activated by aberrant tyrosine kinase activation. Fusion protein BCR-ABL is caused by the t(9;22)(q34;q11) translocation, which encodes the constitutively activated tyrosine kinase. Development of the tyrosine kinase inhibitor imatinib had provided a remarkable improvement in the outcome of BCR-ABL-positive chronic myeloid leukemia (CML) or acute lymphoblastic leukemia (ALL). Although, there still remains a challenge how the leukemia acquires imatinib resistance and progress to blast crisis in CML or relapse in ALL. Identification of new pathways and molecules activated by BCR-ABL offers new insights into the pathophysiology of CML and BCR-ABL-positive ALL, and may also provide further development of molecular targeted therapy. Here, we present the phosphoproteome of leukemic cells measured by HAMMOC-based shotgun proteomics approaches. BCR-ABL-positive leukemic cell lines

were digested with Lys-C and trypsin, followed by HAMMOC to enrich phosphopeptides. NanoLC-MS/MS was performed to obtain peptide-fragmentation spectra, and the data were analyzed to identify peptides and proteins by means of automated database search using Mascot (Matrix Science). We have identified more than 1,800 phosphorylated sites through analysis of BCR-ABL-positive cells. BCR-ABL-positive leukemic cell lines formed a phosphopeptide-cluster in parallel analysis of multiple cell lines suggesting a unique signaling phosphorylation pathway in BCR-ABL-positive cells. We are currently analyzing the changes in phosphorylation status when BCR-ABL signaling was modified. One mechanism proposed for resistance to tyrosine kinase inhibitor in CML is the innate insensitivity of primitive quiescent CML stem/progenitor cells, which account for residual disease and evolve to blast crisis. We are verifying phosphorylation of normal hematopoietic stem cells (HSCs), which data will be informative for phosphorylation status of cancer stem cells. In conclusion, our comprehensive method for identifying phosphopeptides is suitable for experiments to study cell signaling networks, which require the enrichment of many phosphopeptides from real complex mixtures with a wide dynamic range. It can be adapted for a wide range of cancer cells which aberrant phophorylated signaling is involved.

0249

SENESCENCE-ASSOCIATED GENE EXPRESSION CHANGES IN MESENCHYMAL STROMAL/STEM CELLS

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Based on promising experimental studies with mesenchymal stromal cells (MSCs), a variety of clinical applications have been initiated. Extensive propagation to yield enough MSCs for therapy may result in replicative senescence and thus hamper long term functionality *in vivo*. Highly variable proliferation rates of MSCs in the course of long-term expansions under varying culture conditions may already indicate different propensity for cellular senescence. We hypothesized that senescence-associated regulated genes differ in MSCs propagated under different culture conditions. Human bone marrow-derived MSCs were cultured either by a highly efficient two-step protocol or by serial passaging in three different growth conditions. Culture media was either supplemented with fetal bovine serum in varying concentrations or pooled human platelet lysate. All MSC preparations revealed significant gene expression changes upon long-term culture. Especially genes involved in cell differentiation, apoptosis and cell death were up-regulated, whereas genes involved in mitosis and proliferation were down-regulated. Furthermore, overlapping senescence-associated gene expression changes were found in all MSC preparations. Long-term cell growth induced similar gene expression changes in MSCs independent of isolation and expansion conditions. A panel of genes will be presented that might offer a practicable approach to assessing MSC quality with regard to the state of replicative senescence in advance of therapeutic application.

0250

EPIGENETIC INACTIVATION OF THE MIR-34A IN HEMATOLOGICAL MALIGNANCIES

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Background. miR-34a is a transcriptional target of p53, and implicated in carcinogenesis. *Aim.* We studied the role of miR-34a methylation in a panel of hematological malignancies including acute leukemia (AML, ALL), chronic leukemia (CLL and CML), multiple myeloma (MM) and non-Hodgkin's lymphoma (NHL). *Methods.* The methylation status of miR-34a promoter was studied in 12 cell lines and 188 diagnostic samples by methylation-specific PCR. *Results.* miR-34a promoter was unmethylated in normal controls but methylated in 75% lymphoma and 37% myeloma cell lines. Hypomethylating treatment led to re-expression of pri-miR-34a transcript in lymphoma cells with homozygous miR-34a methylation. In primary samples at diagnosis, miR-34a methylation was detected in 4% CLL, 5.5% MM samples, and 18.8% of NHL at diagnosis but none of ALL, AML and CML (P=0.011). In MM patients with paired samples, miR-34a methylation status remained unchanged at progression. Amongst lymphoid malignancies, miR-34a was preferentially methylated in NHL (P=0.018), in particular natural killer/T cell (NK/T cell) lymphoma. *Summary.* Amongst hematological malignancies, miR-34a methylation is preferentially hypermethylated in NHL, in particular NK/T cell lymphoma, in a tumor-specific manner, therefore the role of miR-34a in lymphomagenesis warrants further study.

0251

GLOBAL GENE EXPRESSION REVEALS A SET OF GENES THAT MAY BE INVOLVED IN THE PHENOTYPE OF BETA THALASSEMIA MAJOR AND INTERMEDIA

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Background. Beta thalassemia is one of the most common monogenic diseases that reduce the production of hemoglobin. The homozygote state is generally classified into two types, according to the severity of symptoms: thalassemia major (also known as Cooley's anemia) and thalassemia intermedia. Of the two types, thalassemia major is more severe. Patients with thalassemia intermedia maintain the hemoglobin level between 7-11 g/dL and do not require regular transfusions. The cause for this milder clinical condition may be an association with the hereditary persistence of fetal hemoglobin (HPFH), co-inheritance of alpha-thalassemia and other genetic factors. However, in some cases the same genetic mutation leads to different phenotypes of thalassemia (major and intermedia), suggesting the involvement of unknown genes in metabolic pathways related to disease prognosis. *Aims.* In this study we used Serial Analysis of Gene Expression (SAGE), Subtractive Suppressive Hybridization Library (SSH) and Real Time PCR (RT-PCR) to analyze global gene expression in a $CD34^+$ culture and reticulocyte of two patients homozygous for the same genetic mutation (CD39), but with different phenotypes of beta thalassemia (major and intermedia). In these patients, all the polymorphisms and mutations in the gamma and beta globin gene as well as all other known associated factors were evaluated and no difference was found. *Methods.* Global gene expression was evaluated in the reticulocyte and $CD34^+$ cultures using SSH and SAGE respectively. In this culture, cells were collected on the 10^{th} day, when cell proliferation and hemoglobin production are intense. To identify the genes that were differentially expressed between the SAGE libraries, a $P<0.01$ and fold ≥ 5 were considered as statistically significant. For the SSH libraries the genes that appeared only in one of the libraries were considered as differential. *Results.* The global aspects of the transcriptome were very similar between the patients and a small set of genes was identified as differentially expressed between the two patients. We found 38 genes overexpressed in thalassemia major (TM) and 42 in thalassemia intermedia (TI). Between these, we found several genes related with important metabolic pathways like protein binding (KLHL12, OAZ1), transcription factors (MAFF, EYA3), apoptosis (SEPT14, BCL2L11, CARD8, SMAD4), cell adhesion (OCIAD1), DNA repair (APEX1, MORF4L1, RAD23), heme biosynthesis (FECH, HMBS), alpha globin stabilization (PCBP2) and drug resistance (ABCB10). Some of these genes, such as PCBP2, EYA3, ABCB10 and APEX1 are described for the first time in association with beta thalassemia, and their expression was evaluated by Real time PCR, confirming the results found in the libraries. *Conclusions*. The results indicated that the global aspects of the transcriptome were similar, and that the up and down regulation of a small set of genes could be responsible for the differences in the severity of symptoms observed in these patients. The results of this study may contribute to a better comprehension of beta thalassemia, as well as to the identification of new therapeutic target genes. Supported by FAPESP

Granulocytes, infectious diseases and supportive care

0252

LOW NUMBERS OF PERIPHERAL BLOOD CD19+/CD27+ MEMORY B LYMPHOCYTES AND ALTERED LEVELS OF SERUM IMMUNOGLOBULINS IN PATIENTS WITH CHRONIC IDIOPATHIC NEUTROPENIA

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Background. Chronic idiopathic neutropenia (CIN) is a disorder of granulopoiesis characterized by increased apoptosis of bone marrow granulocytic progenitor cells due to the presence of pro-inflammatory cytokines mainly produced by activated T-lymphocytes. Infections are uncommon in CIN patients but their frequency and severity, when present, do not always correlate with the severity of neutropenia. *Aims.* To investigate parameters of humoral immunity in patients with CIN with special focus on the quantitative and qualitative characteristics of peripheral blood B lymphocyte subsets and serum immunoglobulin levels. *Methods.* We studied 46 patients fulfilling the previously defined diagnostic criteria for CIN and 24 age- and sex-matched healthy controls after informed consent. We initially evaluated the proportions of $CD19^+$, $CD20^+$, $CD21^+$ and $CD22^+$ peripheral blood B cells by flow-cytometry. Further analysis of B cell subpopulations was performed on the basis of expression of CD19, IgD, CD38 and CD27 surface markers for the evaluation of the proportion of $CD19^+/IgD^+/CD27^-$ total naïve cells, $CD19^+/IgD^+/CD38^+$ activated naïve cells, $CD19^+/C27^+/IgD^+$ memory cells and $CD19^+/CD27^+/IgD^-$ classical memory cells. We also evaluated baseline surface expression of CD40 on B cells and CD40Ligand expression on immunomagnetically sorted $CD3^+$ T-cells upon stimulation with PMA plus ionomycin. Finally, we evaluated serum levels of IgG1, IgG2, IgG3, IgG4, IgA and IgM by routine nephelometry. *Results.* The proportion of $CD19^+$, $CD20^+$, $CD21^+$ and $CD22^+$ B cells did not differ significantly between in CIN patients and healthy controls. Similarly, the proportion of total naïve $CD19^+/IgD^+/CD27^-$ and activated naïve $CD19^+/IgD^-/CD38^+$ B cells did not differ significantly between CIN patients and healthy controls. Interestingly, however, the proportion of both memory B cell subpopulations namely the $CD19^+/C27^+/IgD^+$ and $CD19^+/CD27^+/IgD^-$ were significantly lower in CIN patients (0.69±0.08% and 1.29±0.74%, respectively) compared to controls (1.11±0.14% and 1.89±0.94%, respectively; P=0.0046 and P=0.013, respectively). The proportion of CD40 expressing $CD19^+$ cells and the intensity of CD40 expression (expressed as mean fluorescence intensity) did not differ significantly between CIN patients and controls whereas the expression of CD40Ligand on mitogen-induced purified $CD3^+$ T-cells did not differ significantly between patients and controls. These data suggest that the lower proportion of memory B cells in CIN patients is not due to impaired activation of surface CD40 by its cognate ligand. The levels of IgM were higher and the levels of IgA and IgG3 lower in CIN patients compared to controls (P<0.0001, P<0.0001 and P<0.0001, respectively). Interestingly, an inverse correlation was found between the levels of IgM and the proportion of classical memory $CD19^+/CD27^+/IgD^-$ cells in the patients (r= -0,4696, P=0.0034). *Summary/Conclusions.* CIN patients display disturbances of B lymphocytes as shown by the low proportions of memory B cell subsets and the altered immunoglobulin levels. Given that normal B cell differentiation and immunoglobulin production depend not only on intercellular interactions but also on environmental cytokine milieus, we assume that the altered levels of cytokine with a B-cell stimulating effect such as TGF-β and IL-10 previously described in CIN may have a role in the above abnormalities.

0253

ECTOPIC EXPRESSION OF MIR-125B BLOCKS GRANULOCYTIC DIFFERENTIATION BY TARGETING MULTIPLE SIGNALLING PATHWAYS

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Background. Granulopoiesis is a tightly regulated process controlled by interaction of cytokines and lineage-specific transcription factors. miRNAs may provide an additional level of control but the functional role of individual miRNAs for granulocytic differentiation is not clearly defined. *Aim.* The aim of this study was to analyze the functional role of individual miRNAs in granulocytic differentiation. *Methods.* We used 32D cells, a murine IL-3-dependent myeloid progenitor cell line, as a model system to study G-CSF-induced granulocytic differentiation. miRNA-based microarray and miR-qRT-PCR were both used to screen for differentially expressed miRNAs at sequential stages of granulocytic differentiation. For selected miRNAs, gain- and loss-of-function phenotypes were generated using lentiviral gene transfer of pre-miRNAs and antagomiRs, respectively. Proliferation/differentiation kinetics (trypan blue dye exclusion, PI staining), cell morphology (May-Grunwald staining), mRNA expression (Real-time PCR) and immunophenotype (flow cytometry) were evaluated. In addition, miRNA-dependent impact on granulocytic differentiation was analyzed in bone marrow-derived murine lineage negative progenitor cells (Lin^-). Lin- cells were lentivirally transduced, sorted and subjected to clonogenic assays. Colony number as well as cell morphology and expression of myeloid surface markers were assessed. Furthermore, several bioinformatic algorithms (PicTar, miRDB, Target Scan, RNA22) were used to predict putative miRNA targets, and modulation of target expression was evaluated using western blotting. Finally, the impact of validated candidate genes on granulocytic differentiation was assessed by lentivirally expressed shRNAs. *Results.* We identified and functionally analysed several miRNAs (including miR-34a-c, -125b, -155, 181b, 223, 291a, 370) potentially involved in regulation of granulocytic differentiation. Ultimately, we focused on miR-125b since neither overexpression nor antagonization of other evaluated miRNAs affected this process. Stable ectopic expression of miR-125b completely blocked G-CSF-induced granulocytic differentiation of both 32D progenitors and Lin- cells. Flow cytometric analysis of myeloid-related surface markers revealed absence of Gr1 and abrogated CD11b expression in 32D/miR-125b compared to 32D/miR-control cells. Furthermore, overexpression of miR-125b dramatically changed the expression pattern of myeloid-related transcription factors, such as STAT3, C/EBPα, C/EBPε, and PU.1. Consequently, transcriptional profiling of genes encoding primary and secondary granule components (MPO, G-CSFR, LZM, LCN2) revealed significant alternation in their expression. Clonogenic assays of Lin- cells demonstrated that miR-125b cultures generated more and larger colonies as compared to miR-control cells, indicating elevated number of cells per colony. Morphological analysis of cells isolated from methylcellulose revealed a block of granulocytic differentiation of Lin-/miR-125b cultures without blast transformation. In addition, we predicted and evaluated several signalling pathways potentially regulated by miR-125b, such as the BCL-2 family, JAK-STAT and MAPKs. Particularly, BAK1 protein level was significantly reduced by miR-125b overexpression. However, RNAi targeting BAK1 resulted only in a delay but not a block of granulocytic differentiation. *Conclusions.* Although several miRNAs were predicted to play a role in granulocytic differentiation, only stable ectopic expression of miR-125b completely blocks this process in both 32D and primary Lin- cells. BAK1 is a validated target of miR-125b, but reduction of BAK1 protein level alone is not sufficient to mediate the described phenotype. Therefore, additional molecular mechanisms must be involved in miR-125b-mediated block of G-CSF-induced granulocytic differentiation.

0254

INVASIVE FUNGAL DISEASES IN FRENCH PATIENTS WITH HEMATOLOGIC MALIGNANCY OR RECIPIENTS OF HEMATOPOIETIC STEM CELL TRANSPLANTATIONS

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Background. Invasive fungal diseases (IFD) are a major problem and a frequent cause of death in immunosuppressed patients such as neutropenic patients or recipients of hematopoietic stem cell transplantation. *Aims.* The purpose of this work was to describe incident invasive fungal disease, in patients with hematologic malignancy or recipients of hematopoietic stem cells transplantation. *Methods.* Incident and prevalent IFD were collected and documented during an observational prospective study conducted in 37 hematologic facilities scattered across

the French territory. All patients with a suspected IFD were to be included. Information about patients' background disease and treatment, clinical features, medical imaging and mycological results were collected and after the start of any non-prophylactic antifungal treatment (referred to as "day 0"henceforth). IFD diagnosis at day 0 and day 7 were classified according to the 2008 revised definitions from the European Organization for Research and Treatment of Cancer/Mycosis Study Group (EORTC/MSG). Informed consent was obtained from all participants. *Results.* Eighty-one French hematologic facilities were offered to participate, 41 accepted and 37 did include 420 patients, 299 adults and 121 children, between December 2007 and December 2008. Three of these patients experienced two fungal infections during the course of the study, thus 423 episodes are described below. Characteristics of these patients and episodes are summarized on the attached table. At day 0, 75 (18%) episodes were possible mould infections, 59 (14%) where probable or proven mould infections, 30 (7%) were proven yeast infections and 250 (59%) did not meet EORTC/MSG criteria. At day 7, 76 (18%) episodes were possible mould infections, 80 (19%) were probable or proven mould infections (mostly due to Aspergillus spp) and 38 (9%) were proven yeast infections, (mostly due to Candida spp). The remaining 224 (53%) were insufficiently documented to be classified according to EORTC/MSG definitions. Thus mould infections represented 79% of documented fungal infections. *Conclusions.* Overall, 47% of suspected IFD in French hematologic patients are documented by mycology and/or typical clinical or radiological aspects the seventh day of antifungal treatment. Four in five documented fungal infections are mold infections, mostly due to Aspergillus species. The remaining are mostly due to Candida species.

Patients' characteristics and IFD diagnosis at day seven after starting antifungal treatment (n = 420 patients, 423 suspicions of IFD)

	n	(%)
Gender (male)	236	(56%)
Age (≥ 18 years)	299	(71%)
Underlying disease		
Acute myeloblastic or lymphoblastic leukemia	258	(61%)
Hodgkin's or non Hodgkin's lymphoma	53	(13%)
Multiple myeloma	18	(4%)
Myelodysplastic syndrome	16	(4%)
Chronic leukemia	13	(2%)
Aplastic anemia	10	(2%)
Other	52	(12%)
Hematopoietic stem cell transplantation	115	(27%)
Autologous	40	(35%)
Allogeneic	75	(65%)
Diagnosis at day 7 of the episode (EORTC/MSG definitions)		
Proven yeast disease (mostly *Candida spp*)*	37	(9%)
Proven mold disease (mostly *Aspergillus spp*)	12	(3%)
Probable mold disease*	73	(17%)
Possible mold disease	72	(17%)
Possible fungal disease	2	(1%)
Insufficiently documented episode	226	(54%)

IFD, Invasive fungal disease.
* One patient had both a proven *Candida* infection and a probable *Aspergillus* infection

0255

OPTIMIZED RADIOLOGICAL DIAGNOSIS OF HEPATIC CANDIDIASIS DURING THE TREATMENT OF ACUTE LEUKEMIAS

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Background. Intensive treatment of acute leukemias is associated with long-lasting neutropenias carrying a high risk for bacterial and fungal infections. Hepatic candidiasis is a frequent complication in patients receiving intensive chemotherapy for acute leukemia. *Aim.* Hepatic lesions may be detected by computerized tomographic (CT) scans of the thorax or abdomen, but there is no standardized CT protocol for the diagnosis and follow-up of hepatic candidiasis. *Methods.* All patients underwent sequential thoraco-abdominal CT scans using multi-detector-row CT scanners. We retrospectively analysed the size and the volume of hepatic fungal lesions in 24 thoraco-abdominal CT of 20 consecutive patients treated for acute leukemia during arterio-venous (chest CT) and porto-venous phase (abdomen CT). The size of the lesions was correlated with the density values of liver parenchyma and liver veins in the two CT phases. *Results.* The diagnosis of hepatic candidiasis was made after induction chemotherapy (50%), after consolidation therapy (5%), before allogeneic stem cell transplantation (30%), during re-induction chemotherapy (5%), during follow-up after allogenic transplantation (5%), or during best supportive care (5%). The median absolute neutrophil count at the time of radiological diagnosis was 2.47×10^9/L (range 0.06-18.76$\times10^9$/L). At the time of CT scan, four patients (20%) were in neutropenia (ANC 0.06, 0.24, 0.46, 0.56$\times10^9$/L). C reactive protein levels (median 86 ng/L, 1-273) and the alkaline phosphatase levels (median: 162 U/L, 56-785) were clearly elevated, whereas liver enzymes were within the normal range (AST: 28.5 U/L, 8-234, ALT: 26 U/L, 16-96). The mean number of hepatic lesions per patient was 31 (range: 3-105) in the chest and 26 (3-81) in the abdomen CT (P=0.026). The mean total volume of all lesions was 6.45 mL in the chest and 4.07 mL in the abdomen CT representing a 1.6 fold difference between the two CT scans (P=0.008). The total volume of the lesions negatively correlated to the absolute contrast difference between liver parenchyma and liver vein (Pearson correlation, r=-0.62; P=0.002). *Conclusions.* The chest CT provides a superior distinction of hepatic lesions due to a delayed perfusion of the outer rim of the fungal lesions resulting in an extended visibility. The chest CT is superior to the abdominal CT for initial diagnosis and follow-up of hepatic candidiasis.

0256

INFECTIONS DUE TO KLEBSIELLA IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES: RISK FACTORS FOR SEVERE SEPSIS/SEPTIC SHOCK AND MORTALITY

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Background. Klebsiella species are major nosocomial pathogens causing serious infection in neutropenic setting. The aim of this study was to evaluate risk factors for severe sepsis/septic shock and mortality by infection due to klebsiella. *Methods.* This study was conducted in a teaching hospital to evaluate the clinical profile of infection due to Klebsiella species and to determine risk factors for severe sepsis/ septic shock defined according to the criteria of the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference, and risk factors for mortality. *Results.* Between 2005 and 2009 a total of 160 Klebsiella isolates (118 *K.pneumonia*, 28 *K. oxytoca*, 7 *K terrigena*, 7 others species) were collected in 130 patients: 59 (45.4%) acute myeloid leukemia, 56(43.1%) acute lymphoid leukemia,8(6.2%) lymphoma, and 7 (5.3%) aplastic anemia.The median age was 26 years (range, 2-60 years).There was a trend towards higher incidence of isolates during the year 2006 (37.5%, P=0.05).The common sites of the isolates were blood culture (47.5%), stools (30.5%) and perineal lesion (9.5%). Median duration of neutropenia before isolates was 8 days (0-71days).The major clinical symptoms were from the digestive tracts (34%) and the respiratory tract (17.5%). Susceptibility profile to antibiotics revealed that about 53.7% of isolates were Extended-spectrum β-lactamase producing (ESBL)-Klebsiella.Severe sepsis and septic shock occurred respectively in 27.5 and 12.5% of the episodes. Attributed mortality was 17.7% (23/130). By univariate analysis factors associated with severe sepsis/septic shock were (P<0.0001): disease status (CR vs non CR), neutropenia >8 days, respiratory signs, fever > 3 days on antibiotic therapy, concomitant infection, bilirubin > 50 μmol/L, low prothrombin time, serum bicarbonate <18 mmol/L, serum lactate> 2.4 mmol/l, hemoglobin<70 g/L,and proteinemia <55 g/L. For mortality predictive factors were (P<0.0001): respiratory signs, fever > 3 days on antibiotic therapy, concomitant infection, bilirubin > 50 μmol/L, hemoglobin<70 g/L, proteinemia<55g/L, and elevated a PTT. By multivariate analysis factors found to be associated with severe sepsis/septic shock were: disease status (P=0.038), serum lactate >2.4 (P=0.005), fever > 3 days on antibiotic therapy (P<0.0001), isolate from blood culture (P=0.018) and bilirubin >50 μmol/L (P=0.046). Predictors of mortality were: involvement of respiratory tract (P=0.006), ESBL -Klebsiella (P=0.018), concomitant infection (P<0.0001), fever >3 days on antibiotic therapy (P=0.018). *Conclusions.* This study revealed that resistant fever to antibiotic therapy (>3 days), inappropriate initial antibiotic therapy, isolated ESBL Klebsiella, pulmonary infection ,and concomitant infection are the most predictors of mortality in patient with hematological malignancies and Klebsiella infection.

0257

THE USE OF CARDIAC BIOMARKERS IN DETECTION OF CARDIOTOXICITY ASSOCIATED WITH CONVENTIONAL AND HIGH-DOSE CHEMOTHERAPY FOR ACUTE LEUKEMIA

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Background. Cardiotoxicity is a potentially serious complication of hematooncology treatment that can significantly impair patient's quality of life. The greatest risk for development of cardiotoxicity represent anthracyclines (ANT) and high-dose chemotherapy (HD-CT). Various methods including cardiac biomarkers have been recommended for monitoring of cardiotoxicity in oncology. In this context, experience with new potential biomarkers of cardiac injury is very limited. *Aims.* Monitoring of cardiotoxicity of conventional and HD-CT with multiple biomarkers of cardiac injury - glycogen phosphorylase BB (GPBB), heart-type fatty acid binding protein (H-FABP), cardiac troponins (cTnT, cTnI), creatine kinase MB (CK-MB mass), myoglobin. *Methods.* A total of 47 adult acute leukemia patients were studied - 24 patients treated with conventional CT containing anthracyclines (ANT, mean total cumulative dose 463.2±114.3 mg/m^2) and 23 patients treated with HD-CT (myeloablative preparative regimen Bu/Cy2 or Cy/TBI) followed by hematopoietic cell transplantation (HCT). All patients had normal liver and renal functions during the study. Cardiac biomarkers were assessed prior to treatment (before CT/HD-CT), after first CT with ANT, after last CT with ANT in the first group, after HD-CT and after HCT in the second group. Values above the reference range based on a number of cardiology studies and recommended by the manufacturers (Roche, Randox) were considered elevated. The cut-off values for cardiac injury were as follows: 7.30 µg/L for GPBB, 4.50 µg/L for H-FABP, 0.40 µg/L for cTnI, 4.80 µg/L for CK-MB mass and 76.0 µg/L for myoglobin. *Results.* Before CT/HD-CT, all biomarkers of cardiac injury were below the cut-off values in all patients. GPBB increased above the cut-off (7.30 µg/L) in 4 (16.7%) patients after first CT and in 5 (20.8%) patients after last CT with ANT. GPBB increased above the cut-off in 5 (21.7%) patients after HD-CT and remained elevated in 5 (21.7%) patients after HCT. CTnI became elevated (above 0.40 µg/L) in 2 (8.3%) patients after first and last CT with ANT. Both patients with cTnI positivity had elevated GPBB. Other tested biomarkers (H-FABP, CK-MB mass, myoglobin) remained below the cut-offs during the study. *Conclusions.* Our results suggest that GPBB could become a sensitive biomarker for detection of acute cardiotoxicity associated with conventional CT containing ANT and HD-CT followed by HCT. The predictive value for development of cardiomyopathy in the future is not clear and will be evaluated during a prospective follow-up. Based on our data, a larger prospective and multicenter study would be most desirable to define the potential role of new cardiac biomarkers in the assessment of cardiotoxicity in hematooncology.

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0258

IMPLEMENTATION OF A CLINICAL PRACTICE GUIDELINE FOR CHILDREN WITH CANCER PRESENTING WITH FEVER AT EMERGENCY ROOM

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Background. Febrile neutropenia (FN) is a common complication after chemotherapy administration. Patients with FN may develop severe infection, septic shock, and death. To improve the outcome of oncologic children presented with FN, we developed a clinical practice guideline for these patients to receive a rapid evaluation and the shortened time of first dose antibiotic at emergency room (ER). *Aims.* To evaluate compliance of the clinical practice guideline for children with cancer presenting with fever at our ER and the adverse outcomes after using this guideline. *Methods.* We performed a retrospective cohort study of oncologic children presenting with fever at our ER after the clinical guideline was implemented from January 2007 to December 2008 (cases). The control group was the children with cancer who presented with fever during January 2005 to December 2006. We evaluated the compliance of the clinical guideline by recording the time of initial clinical and laboratory assessment and door-to-antibiotic time. The adverse outcomes of the patients including septic shock and death were determined. *Results.* There were 170 febrile episodes after using the guideline. About half (49.4%) of the patients received clinical assessment and laboratory result within 60 min, whereas the antibiotics were administered within 120 min in 80%. The median of the door-to-antibiotic time was significantly decreased after the implementation of the guideline (75 min vs 180 min, P<0.001). Prevalence of septic shock and intensive care unit admission of cases were significantly reduced compared to controls (P=0.011 and 0.016, respectively). No infection associated mortality was found in the intervention group. After using the guideline, length of stay of cases was significantly shorter than controls (P=0.001). *Conclusions*. Using the clinical practice guideline for oncologic children with fever was found to reduce the adverse outcomes and improve survival of these patients.

0259

EARLY DIAGNOSIS, GUIDANCE OF THERAPY AND IDENTIFYING HIGH MORTALITY SUBGROUPS OF NEUTROPENIC PATIENTS WITH SEPSIS: ROLE OF PROCALCITONIN

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Background. early diagnosis and appropriate therapy of sepsis is a daily challenge in neutropenic patients. Sepsis during neutropenia suffer from a lack of specific clinical symptoms and signs, microbiological cultures require time and may not be positive in sepsis patients. Among the newest biomarkers for sepsis, procalcitonin (PCT) has the highest diagnostic accuracy. Plasma PCT level is a marker of the inflammatory response of the human body to a non-viral infection. Elevated values are highly suggestive of bacterial infection with systemic consequences. *Aims.* to assess the potential role of PCT in the early diagnosis and in the prognosis of neutropenic patients with sepsis. *Methods.* we included 148 neutropenic patients: 40 (27%) with systemic inflammatory response syndrome - SIRS (febrile neutropenia without source of infection), 63 (43%) with sepsis (febrile neutropenia with documented infection) and 45 (30%) with septic shock. All patients were treated in the intensive care unite of hematology department with daily monitoring of inflammatory markers (C- reactive protein (CRP) and PCT). Positive blood cultures were recorded. Patients were treated according to the local guidelines for febrile neutropenia with modification according to the microbiological *Results.* SIRS and sepsis definition was done according the ACCP/SCCM - criteria. *Results.* 43/45 (96%), 44/63 (70%) and 14/40 (35%) patients in septic shock, sepsis and SIRS group respectively had positive PCT level (≥0.5 ng/mL) (P<0.0001) (Table 1).

Table 1.

	SIRS (%)	Sepsis (%)	Septic shock (%)	*P*
No. of pts	40 (27)	63 (43)	45 (30)	
PCT ng/ml				< 0.0001
< 0.5	26 (65)	19 (30)	2 (4)	
≥ 0.5 < 2	10 (25)	20 (32)	2 (4)	
≥ 2 < 10	4 (10)	14 (22)	10 (22)	
≥ 10	0	10 (16)	31 (69)	
Blood culture				
+	0	46 (73)	28 (62)	0.23
-	40 (100)	17 (27)	17 (38)	
CRP mg/l				
Mean ± Std.	156 ± 90	179 ± 80	262 ±135	0.15
Range	1- 455	25 – 370	10 – 542	

PCT level was statistically significant when compared between SIRS and sepsis (P=0.02), SIRS and septic shock (P<0.0001), sepsis and septic shock (P=0.001). The mean serum level of CRP was 156 mg/L (±90 standard deviation - SD) in SIRS and 179 mg/L (±80 SD) in sepsis group (Mann Whitney test, P=0.15), while in septic shock group was 262 mg/L (±135 SD) (Mann Whitney test, P<0.0001). Blood culture was positive in 46 (73%) and 28 (62%) patients with sepsis and septic shock respectively (P=0.23). There was a direct correlation between serum level of PCT and positive blood culture (P<0.0001). 2 (3%) patients died in sep-

sis group with PCT > 10 ng/mL, while 18 (40%) patients died in septic shock group (14 with PCT>10 ng/mL and 4 with PCT >2<10 ng/mL) (P=0.012). There was strong correlation between plasma level of PCT and mortality (P=0.001). The sensitivity of PCT was 96% and 70% in septic shock and sepsis respectively. Specificity was 65%. The positive predictive value was 75% and the negative predictive value was 93% in septic shock and 56% in sepsis. Likelihood Ratio (LR) was 1.09 and 1.5 in sepsis and septic shock. The test efficiency was 68% in sepsis and 81% in septic shock. *Conclusions.* daily measurement of PCT for an early and effective diagnosis is recommended in all neutropenic patients in whom sepsis and a systemic inflammatory response is suspected. PCT is also useful in monitoring the course and severity of systemic inflammatory response. Persistently elevated levels of PCT are associated with poor outcome and are viewed as failure of therapy.

0260

HEPATITIS B INFECTION IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES, CLINICO-BIOLOGICAL EXPRESSION AND OUTCOME

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Background. Hepatitis B virus (HBV) infection, with a frequency between 8% and 12% in various European countries, represents a major challenge in patients with hematological malignancies. The immunosupresion induced by the underlying disease or by the treatment, has important impact on the outcome of HBV infection. *Aims.* We aim to analyze the frequency of HBV infection in patients with hematological malignancies, the HBV type, the clinical and biological expression and infection impact on patients evolution, taking into consideration malignancy and HBV infection treatment options. *Methods.* The study was performed on 12 patients with HBV infection admitted for conventional chemotherapy and/or hematopoietic stem cell transplantation (HSCT) of a hematological malignancy: Hodgkin lymphoma (HL), non-Hodgkin lymphoma (NHL), acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) between 2000 and 2009. The tested HBV infection markers were: surface antigen/antibody (HbsAg/HbsAb), "e"antigen/antibody (HbeAg/HBeAb), anticore antibody (HBcAb) total/IgM subtype, HBV-DNA copies level. *Results.* Out of 311 children, 5 (1.6%) were HBV infected. The underlying disease was ALL in 3 cases, B cell-NHL in two cases and HL in one case. From 75 adults admitted for an autologous/allogeneic HSCT 7 (9.33%) had a HBV infection. The hematological malignancy that required a HCST was HL in 3 cases, B-cell NHL in two cases, ALL and AML, each in one case. In all cases the route of infection was unknown. 10/12 patients were active carriers ($HBsAg^+$, HBeAg+/-, HBsAb-, $HBcAb^+$, increased transaminases levels, HBV-DNA≥20000 copies/mL for wild HBV type or ≥2000 copies/mL for pre-core mutant HBV type) and 2/12 patients were inactive carriers. In 6/10 cases the HBV was a wild type and in 4/10 cases a pre-core mutant type. Considering malignancy as a vital priority chemotherapy was started immediately. In 5/7 cases the HBV infection enforced the HSCT delay with 6 to 24 months. The treatment was started with interferon-α (IFNα) in 5 cases, with lamivudine in 5 cases and with entecavir in one case, concomitant with conventional chemotherapy in 10/11 cases. One patient did not receive any antiviral treatment. In 3/5 cases the resistance to IFNα developed after 6-12-18 months of treatment and the lamivudine was started. The lamivudine resistance determined the switch to entecavir in 1/5 cases. In 3/11 treated cases the HBV-DNA became undetectable after 6 months (entecavir), 12 months (IFNα) and 16 months (lamivudine). One patient presented a HBV infection relapse at the lamivudine interruption, one remained HBsAg carrier and one remained HBV negative. None of the patients developed hepatic failure or died from HBV infection complications. *Conclusions.* The HBV infection represents an important comorbidity in patients with hematological malignancy, much frequent in adulthood. In children the use of anti-HBV vaccine starting 1990 seems to have a great influence in reducing the infection rate. One major challenge in the management of these patients is represented by the development of antiviral therapy resistance. In contrast to literature cited evolution, in our study none of the patients developed severe hepatic manifestation.

0261

IMMUNOGLOBULIN LEVELS AND INFECTIONS IN PATIENTS RECEIVING RITUXIMAB EITHER AS INDUCTION OR AS MAINTENANCE THERAPY

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Background. Rituximab is a monoclonal anti-CD20 antibody that has been widely used for more than a decade to treat patients with haematologic malignancies and autoimmune diseases. In the pivotal single agent rituximab trial, hypogammaglobulinemia occurred in only 14% of cases and was not associated with significant infectious complications. However, several infections related to rituximab have been reported in the literature, especially when it is used in combination with chemotherapy or as maintenance therapy although immunoglobulin levels have not been systematically recorded. *Aims.* The objective of this study was to observe the fluctuation of immunoglobulin levels as well as the incidence of infections in patients receiving rituximab. *Methods.* The study included 96 patients (43 men and 53 women; age range: 23-86 years, mean: 64 years) who were treated in the Day Clinic of our hospital for the past two years (January 2008-February 2010). Among these patients 17 were treated with rituximab as a single agent, while 79 patients received rituximab combined with chemotherapy. In the latter group 26 patients were subsequently given rituximab as maintenance therapy for a maximum of two years. Serum quantitative immunoglobulin levels (IgG, IgA, IgM) were measured each time the patients were treated in the Day Clinic and their medical files were reviewed retrospectively in order to record the infectious complications that developed during their treatment. Patients in whom any of the three gammaglobulin components studied was below the lower limit of normal before the commencement of therapy were excluded from the study. *Results.* a. rituximab as a single agent: hypogammaglobulinemia was observed in 7 (41%) cases, but it was rather subtle and involved almost exclusively the IgM component (median value: 0,31g/L with a lower limit of normal at 0,40g/L). No infections were reported. b. rituximab combined with chemotherapy: hypogammaglobulinemia was observed in 66 patients (83%). The most frequently affected immunoglobulins were IgG (n=49) and IgM (n=47), while IgA was low in 24 patients. In patients with hypogammaglobulinemia, the most profoundly affected component was IgM (median value: 37.5% below the lower limit of normal), followed by IgA and IgG (median values: 27% and 17% below the lower limit of normal respectively). Hypogammaglobulinemia persisted throughout the period of maintenance therapy although in two cases it was partially restored. A total of 42 of infections were reported in 31 patients. Among them 22 were attributed to neutropenia. In the remaining 20 non-neutropenic infections, hypogammaglobulinemia was observed in 15 cases (75%). Four non-neutropenic infections developed during maintenance therapy. *Summary/Conclusions.* Rituximab has been used extensively for more than a decade and its efficacy and safety are now well established. However close monitoring of patients and observation of immunoglobulin levels may be necessary in cases where rituximab is combined with chemotherapy. It should be emphasized that increased vigilance is also necessary throughout the period of maintenance therapy (even after the end of it) because hypogammaglobulinemia is present for a long time and may put patients at a high risk of infections.

0262

CYTOMEGALOVIRUS REACTIVATION AFTER AUTOLOGOUS STEM CELL TRANSPLANT FOR B-CELL MALIGNANCIES: SOMETHING TO DEAL WITH. A PROSPECTIVE STUDY ON 92 CONSECUTIVE PATIENTS

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Background. Cytomegalovirus (CMV) reactivation is a frequent complications of allogeneic stem cell transplantation. However, information regarding the incidence and the clinical impact of CMV reactivation after autologous stem cell transplant (ASCT) are lacking. *Aims.* The aim of the study was to assess the incidence of CMV reactivation after autologous stem cell transplantation in lymphoproliferative diseases.

Moreover, we searched for possible pre-transplant variables influencing CMV reactivation after transplant. *Methods.* 92 patients consecutively submitted to ASCT for B-cell malignancies were monitored for CMV reactivation. Twenty-nine patients had multiple myeloma, 63 a lymphoproliferative disease (HD, NHL, CLL). Twenty-seven patients received ASCT for a resistant disease, whereas 65 for a newly diagnosed disease. Thirty-six patients received anti-CD-20 antibody as a part of the frontline therapy before ASCT. The conditioning regimen was BEAM for 47/92 patients, high-dose Melphalan for 28/92 patients and BAVC or Busulphan/Endoxan for the remaining 17 patients. Sixty-six patients received peripheral blood stem cells rescue after high dose therapy, whereas 26 got bone marrow stem cells. CMV reactivations were monitored twice weekly with the polymerase chain reaction (PCR) assay in all patients, starting after the engraftment. *Results.* Twenty-five out of 92 patients (27%) presented a CMV reactivation. The vast majority of patients (17/25, 68%) reactivated CMV after 30 or more days from transplant. Moreover, 15/25 patients (60%) presenting a CMV reactivation had received prior Rituximab. Ten out of 25 patients (40%) presented symptoms such as fever, vomiting, arthralgia or profound asthenia at reactivation. Moreover, CMV reactivation was associated with moderate to severe neutropenia in 8/25 patients. All patients reactivating CMV were treated with intravenous (iv) ganciclovir given 7.5 mg/kg once daily for a median of 20 days (range: 6-40). Discontinuation of antiviral therapy required at least two consecutively negative PCRs performed at least 3 days apart each other. All patients experienced a negativization of CMV-specific PCR after a median of 12 days of therapy (range: 9-34). None of the patient developed CMV disease. Moreover, no late infections or other side effects were observed during the long-term follow-up period. *Conclusions.* Our experience clearly demonstrates that a significant proportion of patients submitted to ASCT develop a CMV reactivation after transplant, frequently symptomatic, requiring antiviral therapy. We underline the necessity of a stringent monitoring of CMV reactivation after ASCT, specially for patients receiving therapy with anti-B lymphocytes monoclonal antibodies before transplant.

0263

VORICONAZOLE OR ITRACONAZOLE FOR PREVENTION OF INVASIVE FUNGAL INFECTIONS AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION (ALLOHCT) - FOCUS ON PATIENT PREFERENCE AND LONG-TERM TOLERABILITY

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Background. The IMPROVIT study compared voriconazole to itraconazole as primary antifungal prophylaxis in alloHCT recipients. Although there was no difference in breakthrough fungal infection rates or survival at 180 days, voriconazole was better tolerated for longer durations in this study. *Aims.* To identify the major reasons for the difference in tolerability between these 2 antifungal agents when used as primary prophylaxis in alloHCT recipients. *Methods.* Data for this analysis were collected in a prospective, randomized, open-label, multicenter comparative study of voriconazole compared with itraconazole for primary prophylaxis in alloHCT recipients (note: neither agent has an approved indication for antifungal prophylaxis). Informed consent was obtained from all patients. Trial design and results have been described previously (Marks D. *et al.* Presented at ICAAC 2009. Abstract M-1249a). However, patient data from 1 study center (10 voriconazole patients and 14 itraconazole patients) were subsequently excluded from all efficacy analyses because of clinical trial conduct issues. Success of antifungal prophylaxis was defined as ability to continue study drug for at least 86 days, with survival free from breakthrough fungal infection for 180 days after alloHCT. Patients completed a Treatment Satisfaction Questionnaire for Medication (TSQM) at day 14, self-evaluating each agent in terms of effectiveness, side effects, convenience, and global satisfaction; a scale score was generated from the responses for each domain, with higher scores indicating greater satisfaction. Treatment group comparisons of continuous variables were performed using the 2-sample t-test. A stepwise Cox regression was used to explore the dependent relationship between TSQM scores and treatment completion status. *Results.* In the revised analysis of this study, 224 patients received voriconazole and 241 received itraconazole as primary antifungal prophylaxis. Treatment groups remained well matched for baseline characteristics. Success of prophylaxis at day 180 was significantly higher with voriconazole than with itraconazole (48% vs 33%; adjusted 95% CI for difference: 8%, 25%; P<0.01). Survival rates at day 180 were similar (84% with voriconazole vs 85% with itraconazole; P=NS), as were the incidences of breakthrough fungal infection (1% vs 2%, respectively). Patients were able to tolerate voriconazole for longer periods compared with itraconazole: 96 vs 68 days, respectively (P<0.01). The most common reason for inability to complete 86 days of study drug prophylaxis was intolerance, which was the case for 22% of itraconazole and 7% of voriconazole patients (P<0.01). More than 60% of patients completed TSQM questionnaires at day 14: TSQM scores were significantly higher (P<0.01) for voriconazole in the domains of effectiveness, convenience, and global satisfaction; no significant difference was observed between voriconazole and itraconazole in terms of patient-assessed side effects (Table). Of note, the global satisfaction score was found to be a significant predictor of a patient's ability to complete at least 86 days of prophylaxis (P=0.02 on Cox regression). *Conclusions.* Reanalyzed data from the IMPROVIT study were consistent with previous findings. The superiority of voriconazole in the primary end point of this study was driven by its better long-term tolerability, which was reflected in higher patient-reported TSQM scores for effectiveness, convenience, and global satisfaction.

Table. Mean TSQM scores at day 14.

TSQM Domain	Voriconazole (N = 224)	Itraconazole (N = 241)	*P* Value
Effectiveness	75 ± 18	68 ± 19	0.0026
(% responders)	(62%)	(62%)	
Side effects	92 ± 19	88 ± 23	0.1728
(% responders)	(63%)	(63%)	
Convenience	75 ± 17	65 ± 21	<0.0001
(% responders)	(66%)	(65%)	
Global satisfaction	71 ± 16	63 ± 19	0.0003
(% responders)	(65%)	(64%)	

0264

CYTOMEGALOVIRUS MAY HAVE A ROLE IN THE PATHOPHYSIOLOGY OF CHRONIC IDIOPATHIC NEUTROPENIA

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Background. Chronic idiopathic neutropenia (CIN) is an acquired granulocytic disorder characterised by a prolonged, unexplained reduction in the number of circulating neutrophils. Increased apoptosis of bone marrow (BM) myeloid progenitor cells due to an inflammatory BM milieu and presence of activated T-lymphocytes have been implicated in the pathophysiology of the disease. The cause of these abnormalities and the trigger of T-cell activation, remain unknown. *Aims.* To evaluate the possible involvement of cytomegalovirus (CMV) and other herpesviruses in the pathophysiology of CIN. *Methods.* BM cells were obtained from posterior iliac crest aspirates from 22 patients fulfilling the previously defined diagnostic criteria for CIN and 11 haematologically normal subjects, after informed consent. Total BM cells as well as immunomagnetically sorted $CD3^+$, $CD14^+$ and $CD34^+$ BM cells and serum from patients and controls were assessed for CMV detection by means of PCR and Fluorescent *in situ* hybridization (FISH). Long-term BM cultures (LTBMCs) were also set up in patients and healthy controls for CMV detection in the adherent cell layer that mirrors the BM microenvironment cells. LTBMCs were also used for functional experiments. Patient and control LTBMC stromal cells were also tested for presence of other herpesviruses, namely Herpes Simplex Virus (HSV)-1/-2, Varicella zoster virus (VZV), Human Herpes Virus (HHV)-7, HHV-8 and Epstein Barr Virus (EBV) by PCR. *Results.* CMV was detected by PCR in BM cells of 50% of CIN patients whereas it was not identified in any subject of the control group. Viremia in the serum was limited

to 1 CIN case. Further investigation for the identification of CMV genome in $CD3^+$, $CD14^+$ and $CD34^+$ BM cells, representing T-lymphocytes, monocytes/macrophages and progenitor cells respectively, and also in stromal cells from LTBMCs, revealed the presence of viral DNA in the latter cell type, exclusively. FISH applied in the adherent (stromal) cells of LTBMCs from CIN patients confirmed the presence of CMV genome in 0.1% of the cell population. Infection of normal LTBMCs with a CMV strain expressing Enhanced Green Fluorescence Protein (EGFP) resulted in viral latency at the stromal cells, whereas CMV could establish a lytic infection at the stromal cells in LTBMCs only when the non-adherent BM cells had been removed. The clonogenic potential of the non-adherent cell fraction in CMV-infected LTBMCs was lower compared to the mock-infected cultures ($P<0.05$). Interestingly, HSV-1/-2, VZV, HHV-7, HHV-8 and EBV genome was not detected in LTBMC adherent layers of patients or controls, indicating the pathophysiologic significance of the CMV identification in patient samples. *Summary/Conclusions.* CMV is frequently detected in BM stromal cells of patients with CIN. We postulate that the CMV latent infection of BM microenvironment cells may affect the growth of haemopoietic progenitor cells and may, therefore, have a role in the pathopysiology of neutropenia in the affected subjects.

0265

LATE ONSET NEUTROPENIA IN VLBW NEONATES

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Background. Late-onset neutropenia is one of the hematological abnormalities of prematurity. It usually emerges after the third week of age and it is triggered by short-term bone marrow failure to respond to increased needs. *Aim.* To evaluate the severity and outcome of late-onset neutropenia in very low birth weight (VLBW) premature neonates. *Methods.* Premature infants with BW<1500 g who developed late-onset neutropenia without any apparent cause were studied retrospectively. All infants were healthy on the onset of neutropenia. *Results.* Late-onset neutropenia was recorded in 35 preterm infants (19 boys) with gestation age 28.7±1.8 weeks and birth weight 1101.1±213.8 g. Fifteen (43%) had gestation age <28 weeks. At the onset of neutropenia, the age was 46.25±16.76 days (range17-121 days), the mean white blood cell count was 5375±1885K/uL and the mean neutrophil count was 872.8±251.8 K/uL with values <1000 K/uL in 14 (40%). On follow up, the absolute neutrophil count decreased to <1000 in 27 (77%, P=0.004) preterm infants. There were no significant differences in absolute neutrophil count between preterm infants with gestation age <28 weeks and those with gestation age 29-32 weeks. Mean duration of neutropenia was 19±2 days (range 2-159 days). A two-phase alleviation of neutrophils occurred in 8 infants, with an interval of 9-41 days. 6 infants developed prolonged neutropenia (up to 159 days). The duration and severity of neutropenia was inversely correlated with gestation age. 9 patients were treated with granulocyte-colony stimulating factor (G-CSF) (1-3 days). In 4 patients neutropenia relapsed following G-CSF discontinuation but spontaneously resolved. No infant developed infection during the late-onset neutropenia. *Conclusions.* Late-onset neutropenia is a benign condition that is not associated with increased morbidity of VLBW premature infants despite the extremely low count of neutrophils which may occur.

0266

IMMUNE DYSFUNCTION IN PATIENTS WITH GAUCHER DISEASE: IMPACT OF DISEASE SEVERITY AND ENZYME REPLACEMENT THERAPY

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*Background.*Gaucher disease (GD), the most common lysosomal storage disorder, is a heterogenous multisystem condition. Recurrent infections have been described as a cause of morbidity. A number of immunologic abnormalities have been demonstrated in GD patients. Objectives: This work aimed to assess humoral and cell mediated immunity and phagocytic function in GD patients in relation to parameters of disease severity and enzyme replacement therapy (ERT) .Methods :The study comprised 16 GD patients, 12 males and 4 females , 4 Type I and 12 type III GD. Their age at study entry ranged between 6 months and 16 years (mean 7.82±4.25 years).Ten patients were receiving ERT (recombinant imiglucerase) and 6 newly diagnosed patients before ERT. Baseline assessment included: complete blood count, coagulation profile , liver and renal function tests. Radiological investigations were performed for bony changes and abdominal ultrasound for organ volumes. Bone marrow examination was done for all patients as well as liver and /or splenic puncture in selected patients. β-glucosidase in leucocytes and plasma chitotriosidase were determined. Serum immunoglobulins (Igs): IgG, IgM ,IgA were estimated by immunoturbidimetry and serum IgE by ELISA .Peripheral blood lymphocyte subsets (CD3,CD19, CD4,CD8 and CD56) were analyzed by flow cytometry. Phagocytic function of polymorphonuclear cells (PMNs) was evaluated by candida killing activity measured by cytomorphological method. *Results.* Polyclonal gammopathy was detected in 8/16 patients (50%). Serum IgM level was elevated in 87.5% (14 out of 16 patients),and 13 patients (81.2%) had elevated IgG level. Monoclonal IgA gammopathy was detected in 9 patients (56.2%) and monoclonal IgE gammopathy in 5 patients (31.2%). Serum levels of all immunoglobulin classes were higher in treated patients compared with untreated patients, the difference was significant for serum IgG ($P=0.04$) and IgA ($P=0.03$). Decreased expression of B-cell marker (CD19) was present in 12 patients (75%). T lymphocyte dysfunction in the form of reduced expression of CD3 was found in 10 patients (62.5%)and inverted CD4/CD8 ratio in 10 patients (62.5%). Natural killer cell marker (CD56) expression was reduced in 9/16 patients (56.2%). Phagocytosis and lytic activity of PMNs were also markedly decreased in 31.2% and 50% of patients respectively. Among treated patients a significant positive correlation was detected between phagocytic and lytic indices and duration of ERT ($r=0.058$, $P=0.03$) . An inverse significant correlation was found between serum IgG and IgA and duration of ERT ($r= -0.61$, $P=0.019$) and ($r= -0.74$, $P=0.014$) respectively. Age, gender and other parameters of disease severity (hemoglobin level, platelets count) were not related to the immune profile as well as the absence or presence of neurological disease (type I versus type III). *Conclusions.* Serum immunoglobulin abnormalities and lymphocyte function defects appear to be common in GD. Markedly compromised phagocytic function was demonstrated that could be improved with regular ERT. Such abnormalities in the immune system could impair host defense, and may be of particular importance in the pathogenesis of recurrent infections in Gaucher patients

0267

COMPARABILITY OF A NEW BIOSIMILAR GRANULOCYTE-COLONY STIMULATING FACTOR WITH ITS REFERENCE PRODUCT

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Background. Approval of biosimilars in Europe is provided on the basis of comparable quality, safety and efficacy with an existing reference product. EP2006 (filgrastim) is a recently approved biosimilar recombinant human granulocyte-colony stimulating factor (G-CSF) which used Neupogen® as its reference product. *Aims.* To compare EP2006 with its reference product by way of physicochemical and biological protein characterisation, pharmacokinetic (PK) and pharmacodynamic (PD) data and evaluation of clinical efficacy and safety. *Methods.* A wide array of standard and advanced analytical tests were performed to characterise protein identity, purity and stability of EP2006 and its reference product, including peptide mapping with UV detection and mass determination, circular dichroism (CD) and NMR spectroscopy, matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry and liquid chromatography electrospray ionisation (LC-ESI) mass spectrometry, gel isoelectric focussing (IEF), cation-exchange chromatography and reverse-phase high performance liquid chromatography (RP-HPLC). Biological characterisation included comparison of G-CSF receptor binding affinity by surface plasmon resonance spectroscopy, an *in vitro* cell proliferation assay, and Western blot immunological binding. A total of 146 healthy volunteers received EP2006 and Neupogen® as subcutaneous (SC) injections of 1 μg/kg (n=24), 2.5 μg/kg (n=28), 5 μg/kg (n=28) or 10 μg/kg (n=40) or as a single intravenous 5 μg/kg dose (n=26) in four double-blind, randomised crossover phase I studies. Absolute neutrophil count (ANC), $CD34^+$ cell count and PK parameters were assessed. Supportive efficacy and safety data were obtained from a single-arm open-label phase III study in which 170 patients with breast cancer undergoing doxorubicin and docetaxel chemotherapy received SC EP2006 (300 or 480 μg daily depending on whether body weight < or ≥60 kg) from day 2 of each chemotherapy cycle. *Results.* The primary structures of EP2006

and Neupogen® were shown to be identical by peptide mapping and other tests. CD and NMR spectroscopy demonstrated that both products have comparable secondary and tertiary structures. RP-HPLC, IEF and other methods showed that both products had a similar impurity profile. Stability profiles and degradation pathways were similar for both products with no aggregates being detected. Comparable affinity with the G-CSF receptor GCSFR/CD114 was obtained using surface plasmon resonance spectroscopy, and comparable *in vitro* bioactivity was shown in a cell proliferation assay. Similar affinity to anti-G-CSF antibodies were shown on a Western blot. In phase I studies, EP2006 and Neupogen® had comparable effects on ANC and $CD34^+$ count with confidence intervals within predefined equivalence boundaries. PK parameters also showed bioequivalence. EP2006 was effective and well tolerated in patients with breast cancer. *Conclusions.* These results show the comparability of EP2006 and its reference product.

0268

CLINICAL AND BIOLOGICAL SPECTRUM OF LARGE GRANULAR L YMPHOCYTE PROLIFERATIONS

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Background. Large granular lymphocytes (LGL) are considered to be activated cytotoxic cells of T or NK lineage and may be observed in various settings ranging from autoimmune, viral or neoplastic disorders. LGL associated with neutropenia and/or arthralgia are considered a distinct entity where a pathogenic role of LGL is suspected. *Aims.* The precise spectrum of clinical and biological manifestations associated with LGL is unclear. We retrospectively reviewed the files of patients followed at our institution for whom an LGL expansion was detected by smear or immunophenotypic analysis. *Methods.* T and NK LGL expansions were systematically explored in patients with unexplained neutropenia, absolute lymphocytosis or LGL detected on blood smear examination. Clonality and immunophenotype were assessed by TCRg PCR and flow cytometry respectively. LGL were considered primitive when no other cause was identified before or during the six months following LGL identification or were otherwise considered secondary. *Results.* We identified 113 consecutive patients followed between 1992 and 2009 (44 males and 69 females). Mean age at diagnosis was 57 years (17.5-88.5). 62 (55%) were primitive and 51 (45%) were secondary. A T-cell phenotype was noted in 92 cases (81%) and NK LGL were noted in 14 patients (12%). A T-cell clone was detected in 59/102 (58%) of cases. Neutropenia was present in 59/113 (52%) and was more common in primary compared to secondary LGL (63 vs 25% respectively, P<10-3). Infections were more common in the secondary group (25% vs. 11%, P<0.05). In patients with neutropenia, mean absolute neutrophils were 0.8 (0-1.6G/L) and was similar in both groups. The average lymphocyte count was higher in the secondary group (5.0 vs 3.3 G/L, P<10-3). Arthralgias were present in only 20 patients (11 primitive and 9 secondary). Clinical and biological manifestations of autoimmunity were present in 12 and 23 patients with primary LGL and 12 and 12 patients with secondary LGL respectively. Absolute count of LGL was neither correlated to neutropenia nor to arthralgias. Among the secondary cases, 40 (78%) had haematological malignancies (14 Myeloma/MGUS, 11 low grade B-cell lymphomas, 2 diffuse large B-cell lymphomas, 7 acute leukemias, 3 myelodysplastic syndromes). Eight patients had received allogeneic stem cell transplantation (7 for acute leukaemia, 1 for myelodysplastic syndrome). In the latter group, the diagnosis of LGL was made after the transplantation procedure. Twelve patients had undergone splenectomy. After a median follow up of 36.5 months (0-195), 7 patients had died, 6 of causes unrelated to LGL and 1 of secondary acute leukaemia following treatment with cyclophosphamide. *Conclusions.* Both primary and secondary LGL are more commonly of T-cell phenotype. Although neutropenia is more frequent in primary LGL it is rarely symptomatic. Neither the presence of a T-cell clone nor the absolute number of LGL correlate with neutropenia or symptoms. A high proportion of LGL are associated with indolent B-cell malignancies neoplasias or splenectomy. Survival does not appear to differ from the general population in primary LGL. This study underscore the indolent course of primary LGL.

0269

NEUTROPHIL APOPTOSIS IN NEUTROPENIC PATIENTS WITH HEPATITIS C INFECTION: ROLE OF CASPASES 3, 10, AND GM-CSF

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Patients with chronic HCV infection are prone to increased susceptibility bacterial infection due to neutropenia complicating the course of this disease.The molecular mechanism of neutrophil apoptosis has not been clearly defined. Neutrophils harvested from neutropenic patients with Hepatitis C infection (n=26), and nine age and sex-matched healthy control subjects were examined for the degree of apoptosis. Neutrophil apoptosis was quantified by flow cytometry through determination of Annexin V expression at 0 time (fresh neutrophil), and 24 hs culture. Neutrophils from healthy subjects were also incubated with either 10% heterologous normal or neutropenic sera with and without 10 ul GM-CSF. Caspase 3, 10 were assessed colormetrically in neutrophils at 0 time and after 24hs culture. At 0 time culture the neutrophil apoptosis of the HCV patients was insignificantly higher as compared to that of normal control (P=0.059). At 24 hours culture, patients neutrophils cultured with their own sera showed neutrophil apoptosis significantly increased as compared to that at 0 time culture and this effect was significantly attenuated in similar culture with addition of GM-CSF (P<0.001). On the other hand patients neutrophil cultured with normal sera showed insignificantly increased neutrophil apoptosis at 24 hs culture as compared to that at 0 time culture. Caspases 3, and10 activities were significantly higher in patients neutrophils after 24hs cultured with patients own sera as compared to 0 time culture (P<0.001 for both). Addition of GM-CSF to the culture down regulates the caspase 3, 10 activities. The correlation study between annexin -V expression and caspases activities revealed a borderline positive correlation between annexin -V and caspase 3 (r=0.376, P=0.058), and significant positive correlation with caspase 10 activity (r=0.494, P=0.01). These findings suggest that enhanced neutrophil apoptosis demonstrated in neutropenic patients with HCV infection is through activation of Caspase 10 and is attenuated by GM CSF.

Lymphoma - Clinical 1

0270

FRONTLINE TREATMENT WITH THE COMBINATION FLUDARABINE-CYCLOPHOSPHAMIDE IN LOW-GRADE NON-FOLLICULAR NON-HODGKIN LYMPHOMA: A LONG TERM UPDATING OF GISL LL02 TRIAL

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Background. Indolent non-follicular lymphomas (NFo-NHL) include small lymphocytic lymphoma (SLL), lymphoplasmacytic lymphoma (LPC), marginal zone lymphoma (MZL). This heterogeneous group show different presenting features, behaviour pattern and treatment outcome. This subset of lymphomas have been relatively poorly investigated, and only retrospective studies or prospective trials involving limited series have so far been published. *Aims.* In 2002 the Gruppo Italiano Studio Linfomi (GISL) initiated LL02 prospective multicenter phase II trial, with the aim to evaluate the efficacy and safety of FC combination as front-line therapy of NFo-NHL patients. *Methods.* Between July 2002 and September 2006, 63 adult patients affected by NFo-NHL in active disease phase, were consecutively enrolled in 12 GISL hematological centres. After histologic revision, 61 patients could be enrolled in the study (36 males and 25 females, median age 64 yrs, range 40-75). The series included 22 cases of SLL, 11 LPC, 25 MZL, 3 CD5 negative NHL cases. Patients were treated with a dose of 25 mg/sqm Fludarabine plus 250 mg/sqm Cyclophosphamide administered intravenously daily for 3 days; each cycle was repeated every 28 days for 6 courses; an intermediate evaluation was performed after the third cycle. During the treatment patients received oral thrimethoprim-sulphametoxazole and fluconazole prophylaxis. *Results.* Two patients were excluded because no further information after registration have been obtained. Six patients were withdrawn before the intermediate evaluation for early toxicity: 2 lethal infective episodes (WHO grade 4), 3 haematological toxicities (WHO grade 3-4) and 1 renal toxicity (WHO grade 4). After the intermediate evaluation, 51/59 patients (86.4%) had an objective response (ORR) with a 22% of complete remission (CR) and 64.4% of partial remission (PR). Among the 53 remaining patients, 43 completed the planned treatment of six cycles, 3 five cycles, 3 four cycles, 2 three cycles and 1 progressed after first cycle. At the final evaluation the ORR percentage was 83% with a 40.6% of CR (24 pts) and 42.3% of PR (25 pts); three patients were in progressive disease (5.0%) and one in stable disease (1.6%). On the basis of intention to treat analysis, after a follow-up of 60 months, the median overall survival (OS) was 64%, the progression free survival (PFS) was 54% and the failure free survival was 39%. The median remission duration was 26 months. After a median follow up of 36 months, mortality was 28% (17/61): among them, it was related to disease relapse/progression (35%), sepsis (29%), second tumor (18%), cerebrovascular event, respiratory insufficiency and other causes (6%). About the toxicity profile, the major toxicity was hematological with a 18% cases of WHO grade III or IV anemia, 34% neutropenia and 11% thrombocytopenia. The 10% of patients had an infective episode of WHO grade III-IV. *Conclusions.* FC chemotherapy is a useful chance for advanced untreated non follicular low-grade NHL, with an optimal ORR, CR and PFS. OS is not significantly improved in comparison with fludarabine alone or with standard therapy, even though the quality of responses was better. Infective (2 early deaths) and haematological toxicity, causing the interruption of the planned treatment in a significant subset of patients, suggest an accurate selection and a careful monitoring during the therapy.

0271

EFFICACY AND SAFETY OF BORTEZOMIB AND RITUXIMAB ASSOCIATION IN RELAPSED/REFRACTORY INDOLENT NON-FOLLICULAR AND MANTLE CELL LYMPHOMA: FINAL RESULTS OF PHASE II STUDY BY INTERGRUPPO ITALIANO LINFOMI

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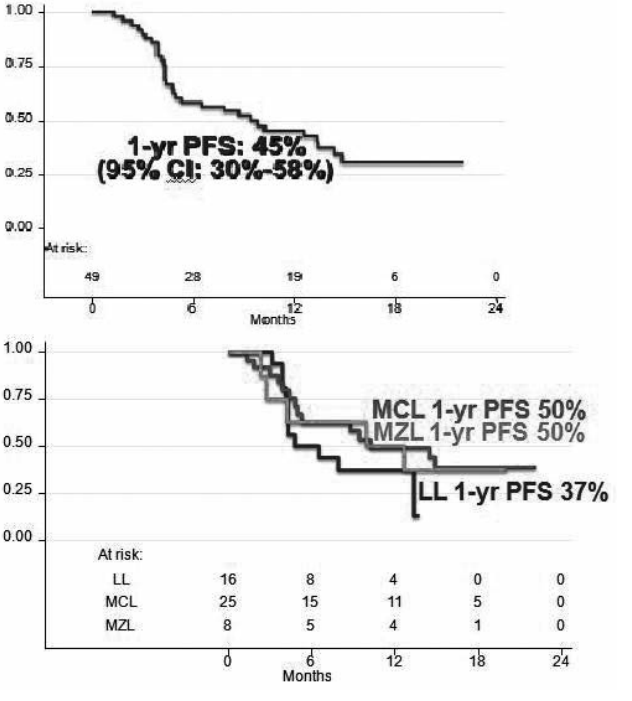
Background. Bortezomib alone or in combination with Rituximab has shown clinical benefit in treatment of Mantle Cell Lymphoma (MCL) and Marginal Zone Lymphoma (MZL). *Aims.* To evaluate safety and efficacy of Rituximab and Bortezomib combination in relapsed/refractory indolent non-follicular lymphoma and MCL not eligible to high-dose chemotherapy. Patients and *Methods.* The study was a phase II multicenter trial according to Simon's design. Inclusion criteria were: age 18-75 years, histological proven relapsed (> 1 year from the last therapy) or refractory (<1 year) indolent non-follicular (linfocytic lymphoma, LL, or MZL) and MCL after 1-4 lines of therapies. Treatment plan was: one course of four weekly intravenous bolus of 1.6 mg/sqm Bortezomib in combination with four infusion of 375 mg/sqm Rituximab followed by two courses of four weekly bolus of 1.6 mg/sqm Bortezomib. Patients with complete (CR), partial remission (PR) and stable disease at the intermediate evaluation were planned to be given three further courses with the same schedule. *Results.* From September 2006 to March 2008, 55 patients entered into the study. Histology revision was performed by three expert pathologists. Forty-nine patients fulfilled inclusion criteria and were evaluable. Clinical characteristics were: median age 68 (50-74) years; 16 LL, eight MZL, 25 MCL; 42 stage III/IV; 33 bone marrow involvement; 20 at intermediate-high/high IPI risk. Thirty-eigh patients performed > two prior lines of chemotherapy; 34 were Rituximab-pretreated; 21 refractory and 28 relapsed disease. Overall Response Rate (ORR) was 53% (CR 26.5%, PR 26.5%); no response 43% and 4% off therapy for other causes. ORR by histology was: 37% in LL, 50% in MZL and 64% in MCL. ORR was not adversely affected by Rituximab pretreatment: Rituximab-pretreated 62% and Rituximab-naïve 33%. ORR was higher in relapsed patients compared with refractory ones: 64% and 38% (p.06). With a median follow-up of one year, Overall Survival was 89% (95%CI: 75-95) and 1-year Progression free survival (PFS) was 45% (95%CI: 30-58) (Figure 1A). One-year PFS was 50% for MZL and MCL and 37% for LL (Figure 1B).

Figure 1.



A total of 233 courses were delivered with a median of 4.7 courses per patient. Thirty patients completed the treatment plan; 19 did not because of progression disease in 13, adverse events in five (one each: concomitant gastric neoplasia, neurotoxicity grade II, sepsis, pleural effusion and toxic death due to interstitial pneumonia). Grade 3-4 CTC haematological toxicity was rare: neutropenia in 5% of the courses and thrombocytopenia in <2%. Grade 3-4 CTC cumulative non-hematological toxicity was observed in 4.7% of all courses. The most frequent non-hematological toxicities were: neurotoxicity grade III in four patients, with complete recover or return to grade I in all of them. Infections were observed in eight patient with ten events: viral reactivation in four, pneumonia in three, sepsis in one and micosis in two. *Conclusions.* The combination of Rituximab and Bortezomib in weekly schedule was effective and safe in treatment of relapsed/refractory indolent and MCL. PFS was promising also in Rituximab-pretreated patients and mainly in MZL and MCL.

0272

TWO YEAR FOLLOW UP OF THE CANADIAN TOSITUMOMAB AND I131 TOSITUMOMAB (TST/I131-TST) EXPERIENCE: THE LARGEST PHASE II STUDY WITH RIT IN INDOLENT NON HODGKIN LYMPHOMA WITH PRIOR RITUXIMAB (R) TREATMENT

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Background. Indolent NHL is characterized by relapses and remissions and tends to respond less to each successive line of therapy. Radiotherapy use in NHL is generally limited to localized disease or palliation, being more toxic as field size increases. Immunotherapy against CD20 has revolutionized NHL specifically targeting B cells. Combining these two modalities as radioimmunotherapy has been shown to be highly active but remains to be fully characterized. *Aims.* Further assess the response rate, the duration of response and toxicity to treatment with I131-T. *Methods.* A phase II open label study of TST/I131-TST was conducted at 12 Canadian centres. Patients with indolent NHL with at least two prior lines of therapy (at least one including R) who had responded to their last treatment were enrolled after obtaining informed consent from April 2004 to February 2007. *Results.* 93 patients were enrolled and included in the intent-to-treat population analysis. Median age was 59 yrs (range 32-78) and 54% were males. ECOG status was 0 in 55.9% and 1 in 37.6%. Follicular NHL was present at original diagnosis in 94.6% of the patients, 78.5% with pathologic grade 1 or 2 histology. Bone marrow involvement was documented in 25.8% of patients. The median duration of disease was 4.9 yrs (range 0.8-22.7). The median number of prior therapies was 5 (range 2-14) with 39.8% of patients enrolled within 6 months of their last therapy. 25.8% had received radiotherapy in prior treatment strategies. Over the 26 week study period, response rate was 40.9% (95% CI 30.8-51.5) including 4.3% with complete response. In 77 patients evaluable for this endpoint, the response rate was 49.4% (95% CI 37.8-61.0). With 2 years of follow up, the median duration of response was 41.4 months (95% CI 22.0-IND, range 4.2-56.4+). Median progression free survival was 12.1 months (95% CI 10.8-17.9). Median overall survival at 2 years was not reached (95% CI 33.3-IND, range 1.2-58.0+ months). There were 38 deaths, 36 of progressive disease, 1 septic shock after progression and 1 respiratory failure. Median FACT-lym scores for quality of life did not significantly decline during the study and improved by week 7 compared to baseline. While on study (to 26 weeks), treatment was well tolerated, 13 patients (14%) had at least one serious adverse event, six considered related to study medication by investigators. 98% of patients had at least one adverse event (AE), the most frequent were fatigue (49%), nausea (43%), cough (31%), headache (23%) and diarrhea (20%). 33% had a grade 3/4 AE, including neutropenia (1%/5%), anemia (3%/0%) and thrombocytopenia (0%/1%). One febrile neutropenia was observed. *Conclusions.* Treatment with TST/I131-T was associated with durable responses with no unexpected toxicities in this heavily pretreated large cohort of patients. A multi-variable model will be explored for predictors of response and other study endpoints; a preliminary stratification by factors including age (<70), number of prior therapies (<4) and size of greatest lymph node (<7 cm) identified cohorts of patients with greater response rates.

0273

OFATUMUMAB IN COMBINATION WITH CHOP (O-CHOP) ACHIEVES HIGH RESPONSE RATES IN PREVIOUSLY UNTREATED FOLLICULAR LYMPHOMA (FL) PATIENTS WITH HIGH-RISK FLIPI SCORES

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Background. Ofatumumab is a novel human antibody that targets a membrane-proximal epitope encompassing both the small- and large-loop on CD20, and elicits more rapid and efficient *in vitro* complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity compared with rituximab. Single-agent ofatumumab has previously demonstrated clinical activity in patients with relapsed/refractory FL at doses up to 1000 mg. *Aims.* We report initial results of an open-label, randomized, two-dose, parallel-group Phase II study of ofatumumab in combination with CHOP (O-CHOP) in patients with previously untreated FL. *Methods.* A total of 59 patients with previously untreated CD20$^+$ FL (Grade 1-3, stage III-IV or bulky stage II) were randomized to ofatumumab Day 1, 500 mg (Group A) or 1000 mg (Group B); cyclophosphamide Day 3, 750 mg/m^2; doxorubicin Day 3, 50 mg/m^2; vincristine Day 3, 1.4 mg/m^2; and prednisone Days 3-7, 100 mg every 3 weeks for 6 cycles. The first ofatumumab dose was 300 mg for both groups. One patient withdrew before treatment and was excluded from analysis. The primary endpoint was overall response rate (ORR; 1999 International Working Group criteria) assessed by an Independent Endpoint Review Committee. *Results.* Twenty-nine patients were treated in each group; 93 and 100% of patients in Groups A and B, respectively, received all 6 cycles of therapy. Baseline characteristics for Group A / Group B were: median age, 55/54 years; FLIPI score 3-5, 34% / 38% of patients. ORR (95% CI) was 90% (73, 98%) in Group A and 100% (88, 100%) in Group B (Table).

Table.

Baseline characteristics and response		
Characteristic	**Group A (N=29) OFA 500 mg**	**Group B (N=29) OFA 1000 mg**
Median age (range), years	55 (25–74)	54 (33–73)
Median duration of FL (range), years	0.1 (0–3.6)	0.1 (0–1.1)
Ann Arbor stage[a], n (%) III–IV	24 (83)	26 (90)
FLIPI score[a], n (%) Low risk (0–1) Intermediate risk (2) High risk (3–5)	7 (24) 12 (41) 10 (34)	10 (34) 8 (28) 11 (38)
Patients completing all 6 cycles, n (%)[b]	27 (93)	29 (100)
Response Rates		
ORR, n (%) CR+CRu, n (%)	26 (90) 20 (69)	29 (100) 16 (55)
CR+CRu by FLIPI score, n (%) Low risk (0–1) Intermediate risk (2) High risk (3–5)	 4 (57) 8 (67) 8 (80)	 6 (60) 2 (25) 8 (73)
[a]At screening; [b]Omission/reduction of vincristine in 21 and 34% of patients in Groups A and B, respectively.		

Complete response (CR) + unconfirmed CR (CRu) was 69% in Group A and 55% in Group B. Overall, 16 of 21 patients (76%) with FLIPI score 3-5 attained CR/CRu. Median follow-up was 9.7 months. Ofatumumab infusion-related reactions were primarily grade 1-2 and decreased in incidence with continued therapy; 2 patients (Group B) had

grade 3 reactions during the first dose (infusion-related hypersensitivity reaction, largyngeal edema), but were able to receive subsequent doses. The most common investigator-reported grade 3-4 adverse events were leukopenia and neutropenia (31 and 28%, Group A; 28 and 17%, Group B). Incidences of grade 3-4 leukopenia and neutropenia by laboratory assessments were 83 and 90% in Group A and 72 and 90% in Group B, respectively. Grade 3-4 infections occurred in 10% of patients in Group A and 3% in Group B, with febrile neutropenia in 7 and 3% of patients, respectively. No deaths have been reported at the time of analysis. *Summary/Conclusions.* O-CHOP achieved high response rates, was effective across all FLIPI risk groups and was well tolerated with no unexpected toxicities in patients with previously untreated FL. No differences in toxicity or response were observed between the two dose groups. Longer follow-up time is needed for analysis of survival endpoints.

0274

ENZASTAURIN IN PATIENTS WITH FOLLICULAR LYMPHOMA: PRELIMINARY RESULTS OF A PHASE II STUDY

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Background. Protein kinase C beta (PKCβ) has a pivotal role in normal B-cell signaling and survival. Overexpression of PKCβ is implicated in the pathogenesis of follicular lymphoma (FL). Enzastaurin (ENZ), an oral serine/threonine kinase inhibitor, targets the PKCβ and PI3K/AKT pathways to suppress tumor cell proliferation and angiogenesis, and induce apoptosis. *Aims.* The primary objective of the study is to evaluate tumor response rate (RR) of ENZ in patients (pts) with grade 1 or 2 FL. The secondary objectives are progression-free survival, time to response, duration of response (DoR), and safety. *Methods.* This is an open-label, single-arm, phase II study of pts with histologically confirmed grade 1 or 2 and stage III or IV measurable FL. Pts were either chemonaive or had relapsed after one chemotherapy regimen or single-agent rituximab (completed ≥6 months before enrollment). Pts received 500 mg oral ENZ, once daily (1125-mg loading dose on day 1) for up to 3 yrs or until progression, withdrawal, or unacceptable toxicity occurred. *Results.* A total of 66 pts (22 males and 44 females) with a median age of 62 yrs (range, 33-82 yrs) were enrolled. According to the Follicular Lymphoma International Prognostic Index (FLIPI) score at baseline, 12.1% had low-risk disease (0-1 risk factors), 53.0% had intermediate-risk disease (2), and 34.8% had high-risk disease (3-5). Forty-six pts (70%) were chemonaive and 20 pts (30%) had one prior chemotherapy (including rituximab). As of August 2009, the median overall exposure was 10.1 months (range, 1-26.6 months). Sixty-four pts received ≥1 dose of ENZ and were evaluable for efficacy. Overall RR was 25% (1 complete response, 15 partial responses). Table 1 shows RR by FLIPI score and previous therapy.

Table 1.

	N	Responders, n (%)	95% CI
FLIPI score (risk Factors)			
Low (0-1)	8	1 (12.5)	0.3–52.7
Intermediate (2)	34	10 (29.4)	15.1–47.5
High (3-5)	22	5 (22.7)	7.8–45.4
Prior chemotherapy			
Chemonaive	44	10 (22.7)	11.5–37.8
Prior therapy	20	6 (30.0)	11.9–54.3

CI=confidence interval; N=total number of evaluable patients; n=number of patients.

A total of 25 pts (39.1%) had progressive disease (PD); 60% were progression-free for ≥12 months. The median time to response for responders was 115.5 days (range, 52-327 days). The median DoR has not been reached (range, 59-687 days; 11 pts in remission). Thirty-seven pts (56%) discontinued treatment: 3 pts (4.5%) due to adverse events, of which 2 (3%) were possibly drug-related (bronchitis and skin rash). Ten pts (15.2%) had grade 3 toxicities, one of which was study drug-related (bronchitis). There were no drug-related deaths. Twenty-nine pts (44%) continue receiving treatment. *Conclusion.* Preliminary results indicate that ENZ has clinical activity and is well tolerated in pts with grade 1 or 2 FL. Updated data will be provided at the presentation.

0275

MANAGEMENT OF RELAPSED OR REFRACTORY FOLLICULAR LYMPHOMA PATIENTS - A FRENCH PHARMACO-EPIDEMIOLOGICAL STUDY: THE OLYMPE STUDY (ML20248)

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Background. There is no consensus on the optimal therapeutic strategy for relapsed follicular lymphoma (FL) patients. Treatment includes immuno-chemotherapy alone or followed by either Autologous Stem Cell Transplantation (ASCT), radio-immunotherapy or rituximab (R) maintenance. *Aims.* To describe, in routine practice, treatment modalities and response rates of relapsed FL in a French cohort study. *Methods.* Prospective, multicenter, observational study in patients with histologically confirmed, $CD20^+$ relapsed FL (Grade 1 to 3a) followed for up to 5 years. Eligible patients required a treatment, regardless of what they had previously received. This interim analysis focuses on induction therapy after relapse. *Results.* From January 2007 to September 2009, 260 patients with relapsed FL treated in 39 French Oncology or Hematology Centers were included in the OLYMPE study. Fourteen patients were excluded from this analysis due to either inclusion criteria violation or missing data. Median age was 61 years (range 30-87), 55.3% were male, with Ann Arbor stage I/II (20.2%) or stage III/IV (68.9%) and 62% of patients had high tumor burden according to the GELF (Groupe d'Etudes des Lymphomes Folliculaires) criteria. Most patients were in first relapse 56.9% (n=140), 22.8% (n=56) in second relapse and 20.3% (n=50) in third or more relapse. Previous treatment included rituximab with or without chemotherapy in 53% (n=131) of patients, high dose therapy with ASCT (HDT + ASCT) in 2.4% (n=6) including 1.6% (n=4) with HDT + ASCT in first line treatment. At inclusion, 94% of patients were treated with R + chemotherapy or R monotherapy. The most common chemotherapy regimens used were fludarabine-based chemotherapy (20.4%; mainly FCR 14.3%), CHOP (18.4%), platinum based chemotherapy (15.9%) and CVP (7.5%). At the end of relapse induction treatment, the overall response rate (ORR=CR+PR) was 52.4%, and complete response rate was 32% (whatever the number of prior treatment lines received), see Table 1.

Table 1.

Response rate	CR (%)	PR (%)	ORR (%)	PD/SD (%)
R+ chemotherapy (n=140)	40.7	20.0	60.7	6.4
R monotherapy (n= 52)	21.2	30.8	52.0	11.5
Chemotherapy alone (n=13)	15.4	30.8	46.2	15.4
R+ radioimmunotherapy (n=10)	40.0	10.0	50.0	10.0

Twenty patients were not evaluated at the end of the induction therapy for relapsed disease. *Conclusions*. In routine clinical practice, the most frequently prescribed induction treatment in France for relapsed FL patients is rituximab with or without chemotherapy, for both first and subsequent relapses. Even in this difficult-to-treat population, complete or partial responses were achieved in 52.4% of patients. Further follow-up is needed to assess the treatment strategies used after induction treatment (HDT + ASCT, rituximab maintenance or observation) as well as safety data.

0276

T(14;18) IN FOLLICULAR LYMPHOMA: A REAL- TIME (PCR) FOR A REAL LIFE….?

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Background. T(14;18) is a molecular marker of follicular lymphoma (FL). Polymerase chain reaction (PCR) permits detection of this specific breakpoint. The presence of t(14;18)-bearing cells is assumed to correlate with lymphoma activity. To date, however, the value of t(14;18) assessment on the initial staging and prognosis remains controversial. The quantitative analysis using real-time PCR (RQ PCR) could be more helpful. *Aims.* We performed a very complex testing of t(14;18) including quantitative analysis in blood, marrow or lymph node to assess its prognostic significance. *Methods.* Totally, 202 (160/202 stage III/IV) FL patients were tested for t(14;18) between 03/2001 and 01/2010. Initially, peripheral blood, bone marrow and lymph node tissue were tested if available. Nested PCR (for MBR and mcr) and real-time PCR (RQ PCR) in MBR+ cases were used. Blood and marrow samples were taken before and after therapy. Rituximab with chemotherapy were administered in the absolute majority of cases. *Results.* At the time of FL diagnosis/relapse (151/51), 113/202 (66%) patients were t(14;18) positive using PCR methods, but only 95/202 (47%) were MBR+. Quantitative analysis according to compartment showed the highest numbers of t(14;18) copies/106 CE (cell equivalent) in infiltrated lymph nodes (n=21; median 1.25×10^6, range 340000-8.7×10^6) compared to bone marrow (n=55; median 9800, range 0-3.6×10^6) and peripheral blood (n=58; median 2994, range 0-1.26×10^6) (P<.05). For prognosis evaluation, 75 PCR+ patients with complete molecular and clinical evaluation (median follow up 37 months; range 7-102) were analyzed according to achievement of molecular remission after therapy. Post-treatment PCR-negative subgroup (n=55) showed better prognosis vs. PCR+ subgroup (n=22) with significantly fewer clinical relapses (24% vs 55%), lymphoma-related deaths (5% vs. 40%), and longer PFS (median 21 vs. 12 months; P<012). There were no differences between subgroups in FLIPI, age, proportion of relapsed patients, stage, grade and follow-up duration. Interestingly, the post-treatment PCR- and PCR+ subgroups showed different pre-treatment numbers of t(14;18) copies/106 CE in peripheral blood with median 709 (0-650616) vs. 6870 (114-$1,2\times10^6$) copies as well as in bone marrow with median 2276 (1-451066) vs. 81414 (58-$1,05\times10^6$). These differences were statistically significant p.009 and p.005, respectively. No differences were observed in lymph node compartment. The significance of "molecular burden " before treatment in peripheral blood and/or bone marrow was confirmed by comparison of patients with "poor"and "good"clinical outcome (defined as EFS<20 vs.≥36 months). Patients with "good"outcome (n=20) showed significant lower numbers of t(14;18) copies compared with "poor"prognosis patients (n=16) in peripheral blood (P.002) and bone marrow (p.009), no differences were found in lymph node compartment. No differences in FLIPI, age, stage, grade or proportion of relapsed patients between prognostic subgroups were observed. *Conclusions.* In spite of limited utility of PCR detection of t(14;18), we conclude, that the quantity of t(14;18) in peripheral blood and/or bone marrow at diagnosis or relapse mirrors "molecular tumor burden"and seems to provide a new prognostic toll independent on FLIPI, other clinical or morphological parameters.

0277

RITUXIMAB AND CHLORAMBUCIL AS FRONT-LINE TREATMENT FOR FOLLICULAR LYMPHOMA: EXTENDED DISEASE FREE SURVIVAL WITHOUT INCREASED TOXICITY

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Background. follicular lymphoma (FL) is one of the most common B-cell non-Hodgkin lymphoma (NHL) and is characterized by an indolent course with a continuous relapse pattern. The anti-CD20 monoclonal antibody Rituximab (R) in combination with chemotherapy is now considered a standard first line treatment for FL. This combination significantly improves progression-free survival (PFS), overall response rate (ORR) and complete response (CR) rate in FL compared with chemotherapy alone. *Aim.* here we report our experience with the combination of Rituximab and Chlorambucil (Chl) in untreated FL pts. *Methods.* since November 2001, 58 pts (28 male and 30 female) with FL received R-Chl as first-line treatment. Patients started with an induction phase with four weekly infusions of Rituximab at 375 mg/sqm and 6 consecutive weeks of Chl at 6 mg/sqm/daily. After restaging, all patients with stable or responsive disease proceeded to a consolidation phase with 4 monthly Rituximab infusions and 14 days of Chl each month. Median age at diagnosis was 56 years (range 29-79). Ann Arbor stage was advanced (stage III-IV) in 44 pts (76%) and 16 pts presented an extranodal localization. Only 9 patients were symptomatic. Histological grading was available in 52 cases, and was 1 in 13 pts, 2 in 33 pts and 3 in 6 pts. FLIPI was evaluable in 55 pts and 31 pts (56%) were at low risk. *Results.* after the induction phase, ORR was 98%, with 15 pts in complete remission (CR) and 42 in partial remission (PR). After the consolidation phase, 46 pts obtained a CR and 11 PR; one remained in stable disease. The mean daily dose of Chl received during the induction phase was 10 mg, while in the prolonged treatment was 8 mg. Chl dose was reduced in 25 pts (43%); however, only 13 pts (22%) had a G3-4 neutropenia, and Chl treatment was stopped in only a patient for a persistent G3 neutropenia after the first consolidation cycle. One HBsAg positive patient did not concluded the treatment because of AST/ALT elevation. With a median observation time of 31 months (range 7-129) from the diagnosis, 40 patients still in CR and 5 in PR . Ten patients (17%) relapsed, with a median time to progression of 19 months (range 7-66) and one died because of lymphoma. *Conclusions.* our preliminary results described a safe and feasible combination of immuno-chemotherapy in untreated FL pts. In terms of efficacy, our clinical results seems similar to those obtained with more aggressive therapy, but with a lower toxicity and an easier management. Our experience suggests, that the addition of Rituximab seems to delete all differences depending on the chemotherapy regimen used. Our combination with Rituximab and Chlorambucil may be considered a valid first-line therapy, especially in FL patients not eligible for more aggressive chemotherapy regimens.

0278

PRIMARY BONE MARROW MARGINAL ZONE LYMPHOMA (PBMMZL): A NEW DISEASE ENTITY?

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Background. The present classification of marginal zone lymphomas (MZL) is probably inadequate to describe all the clinical spectrum of the disease. *Aim.* To describe what we consider as a new marginal zone lymphoma category with primary involvement of the bone marrow and blood, without splenomegaly, lymphadenopathy or MALT involvement. *Patients and Methods.* The definition of PBMMZL was based on the following criteria: Infiltration of BM and blood by lymphoma cells with morphologic and immunophenotypic features consistent with marginal zone origin, absence of lympadenopathy, splenomegaly (by clinical and

imaging methods) or other extranodal disease localization. Between 1986-2009, among 213 patients (pts) with MZL, 23 were retrospectively recognised as fulfilling the criteria for their entry into the present analysis, thus representing 9% of the total MZL group in our Departments. *Results.* The main clinical features are presented on table 1. Circulating lymphoma cells were characterized by an heterogeneous morphology, consisting of a mixture of small lymphocytes, monocytoid and villous lymphocytes. They expressed moderate to strong CD20, more often κ light chain (70%), while they were negative for CD5 and CD10 antigens. CD11c and CD23 antigens were expressed in 43% and 48% respectively, while the expression of CD25 (17%) and CD38 (4%) were less common. A highly variable degree of BM infiltration was noted (10-90%, median 25%), while the pattern of BM infiltration was as follows: mixed 39%, nodular 35%, interstitial 13%, diffuse 9% and paratrabecular 4%. Intrasinusoidal infiltration was evident in 2 cases along with nodular infiltration. Immunohistochemistry showed strong expression of CD20 and negativity for CD10, CD5, cyclin- D1 and CD138 antigens. At diagnosis only one pt received therapy due to anemia. During follow up time 2 pts progressed, at 12 and 84 months, with increasing lymphocytosis, without evidence of splenomegaly or lymphadenopathy. These two pts were treated with rituximab and complete remission was achieved. At a median follow up time of 20 months (3-120) no deaths were recorded. *Conclusions.* We are describing a new category of MZL with primary BM involvement, which is characterized by an indolent clinical course and long survival without need of treatment in the vast majority of patients. Despite many similarities with splenic MZL, no pt in our study has so far displayed splenomegaly.

Table. Features of the 23 pts with PBMMZL at diagnosis.

Features	*No pts*	*%*
Age: Median 65y (50-83)	23	100
Male gender	10	43
B-symptoms	0	0
BM involvement	23	100
Performance Status 0	23	100
Stage IV	23	100
LDH elevated	2	9
Hepatitis C	0	0
IPI: Low / Low-Intermediate/High-Intermediate	6 /16 /1	26 /70 /4
Anaemia (Hb<12g/dl)	4	17
Thrombocytopenia (<100x10^9/l)	0	0
Lymphocytosis (>4x10^9/l)	16	70
Clonal B lymphocytes (immunophenotype-blood)	22	95
Paraproteinemia	7	30
Autoimmune phenomena	0	0

0279

STAGE IV MARGINAL ZONE B-CELL LYMPHOMA- DO THEY HAVE DIFFERENT PROGNOSIS? AND, HOW TO IMPROVE ROLE OF CHEMOTHERAPY: CONSORTIUM FOR IMPROVING SURVIVAL OF LYMPHOMA (CISL) STUDY

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Background. Stage IV marginal zone B-cell lymphomas (MZLs) are present in more than one fourth of patients. But, according to previous study, they had a different prognosis between multiple mucosa-associated lymphoid tissues (MALT)-organ involved stage IV and bone marrow (BM) or nodal involved stage IV. *Aims.* In this study we conducted retrospective analyses of the clinical features and treatment outcomes of specific stage IV MZL to identify their clinical features, treatment, and prognosis. *Methods.* For analysis, between Feb. 1996 and Jun. 2009, a total of 94 patients with histologic diagnosis of stage IV-MZL from 17 different institutions in Korea were included. Stage IV was defined MZL involved (i) over 1 MALT organs (M-MZL) or (ii) BM (BM-MZL) or (iii) liver (L-MZL). *Results.* The male/female ratio of the 94 patients was 60 to 34. The median age of our subjects was 59 years (range: 12-82 years). Multiple-MALT organs involved MZL with or without lymph node (LN) was 34 patients (36.2%). BM involved stage IV MZL was 52 patients (55.3%). Median time to progression (TTP) was 2.3 years (95% CI, 2.0-2.3 years). Cause-specific overall survival (OS) was not reached median value. 5 year and 10 year OS rate were 85.2% and 80.7%, respectively. M-MZL and BM-MZL / L-MZL did not show the difference in median TTP (2.4 years versus 2.3 years, P=0.439). But, in the patients who involved LN in stage IV MZL appeared to be associated with a worse prognosis in TTP regardless of M-MZL or BM-MZL / L-MZL (P=0.010). 89 patients had treated - 2 follow up loss and 3 watchful wait. 83 patients had received chemotherapy based treatment. Out of them, CR or PR was achieved in 63 patients (75.9%). Chemotherapy treatment without operation or radiotherapy was done in 62 patients. Evenly 31 patients treated with rituximab included regimen (R+CTx) and with rituximab not included regimen (R-CTx). R+CTx had been shown better response than R-CTx (83.9% versus 54.8%, P=0.026). But, there was no difference in TTP duration (2.0 years versus 1.8 years, P=0.686). *Conclusions.* Stage IV MZL tends to be an indolent disease - characterized by prolonged survival with frequent relapses. The majority of them was controlled well with chemotherapy based treatment, and could achieve prolonged survival. LN involvement was more valuable prognostic factor than M-MZL or BM-MZL / L-MZL for TTP. Rituximab looks like contribution to better response but not in TTP. So we should consider about maintenance treatment of MZL for extend their response duration.

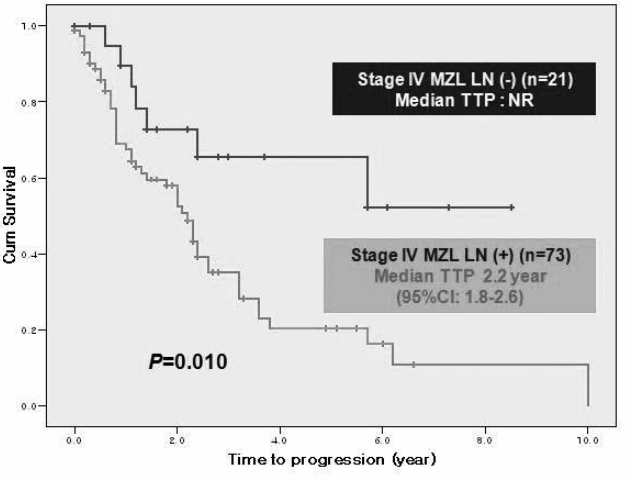

Figure. LN involvement might be a poor prognostic factor

0280

CLINICAL AND VIROLOGIC FEATURES, TREATMENT-RELATED TOXICITY AND OUTCOME OF NON-HODGKIN'S LYMPHOMAS ASSOCIATED WITH HEPATITIS C VIRUS INFECTION: A MULTICENTER ITALIAN STUDY ON 643 PATIENTS

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Background. Hepatitis C virus (HCV) has been associated with B-cell non-Hodgkin's lymphomas (NHL), especially marginal zone lymphoma (MZL) and diffuse large B cell lymphoma (DLBCL). Treatment of HCV-positive NHL is a major issue in the clinical practice: particularly, prevalence and severity of liver toxicity of (immuno)-chemotherapy are not fully elucitated. *Aims.* to analyse clinical characteristics, virological features, treatment-related hepatic toxicity and outcome of a large series of indolent and aggressive NHL associated with HCV infection. *Methods.* we studied 643 HCV-positive patients (pts) with NHL diagnosed and treated from 1993 to 2009 in 16 italian hematologic insititutions. All pts were HIV negative. *Results.* 380 cases were aggressive NHL (373 DLBCL), 263 indolent NHL (114 MZL). Features of pts are summarized in Table 1.

Table 1. Clinical and virus-specific features at presentaion in 643 patients with HCV+ NHL.

	All cases		Aggressive NHL		Indolent NHL		p
	N	%	N	%	N	%	
Age, median (range)	66 (18-88)	-	66 (18-88)		66 (19-87)	-	0.630
M/F	281/362	44/56	174/206	46/54	107/156	41/59	0.199
DLBCL	373	58	373	98			
PTCL	7	1	7	2			
MZL	114	18			114	43	
Splenic	*44*	*7*			*44*	*17*	
Nodal	*22*	*3*			*22*	*8*	
MALT	*48*	*8*			*48*	*18*	
FL	60	9			60	23	
LPL	21	3			21	8	
MCL	18	3			18	7	
SLL	11	2			11	4	
Low-grade B-NHL NOS	39	6			39	15	
Ann Arbor stage I-II	191	30	131	34	60	23	0.001
III-IV	452	70	249	66	203	77	
BM involvement	235	36	79	21	156	59	<0.001
Splenic involvement	239	37	139	37	100	38	0.710
Liver involvement	76	12	52	14	24	9	0.078
B symptoms	160	25	120	32	40	15	<0.001
ECOG ≥2	111	18	89	24	22	9	<0.001
Extranodal sites 1	268	42	169	45	99	38	0.005
≥2	244	38	124	33	120	46	
LDH* elevated	246	42	179	53	67	27	<0.001
IPI* low/low-int	287	52	154	48	133	56	0.030
high-int/high	272	48	163	52	163	44	
HCV genotype* 1a	9	6	4	6	5	6	0.794
1b	56	35	25	36	31	34	
2a/2c	79	49	32	47	47	52	
3a	10	6	4	6	6	7	
4a	4	3	3	4	1	1	
6	1	1	1	1	0	0	
HCV-RNA positive*	380	91	214	92	166	91	0.434
Cryoglobulins*	66	13	23	8	43	18	<0.001
HBsAg-positive	12	2	7	2	5	2	0.900
AntiHBc-positive	167	35	92	33	75	37	0.390
ALT elevation at baseline*	261	42	150	40	111	43	0.482
>ULN-2,5 ULN	185	71	106	71	79	71	0.970
>2,5-5 ULN	58	22	34	22	24	22	
>5 ULN	18	7	10	7	8	7	

NHL Non-Hodgkin's lymphoma, DLBCL Diffuse large B-cell lymphoma, PTCL Peripheral T-cell lymphoma, MZL marginal-zone lymphoma, FL Follicular lymphoma, MCL Mantle-cell lymphoma, LPL lymphoplasmacytic lymphoma, SLL Small Lymphocytic lymphoma, ULN upper limit of normal **Data not available for all patients*

Nine out of 38 HCV-RNA negative pts cleared HCV by means of antiviral therapy before NHL diagnosis. 587 pts received (immuno)-chemotherapy as first-line treatment. Other treatments were as follows: watch-and-wait policy in 25 pts, palliative chemotherapy in 7, splenectomy in 7, anti-HP eradicating therapy in 3 gastric MZL, anti-HCV antiviral therapy in 14 pts (7 of whom obtained both a virologic and hematologic response). Among 587 pts treated with (immuno)-chemotherapy, 369 received CHOP-like regimen (+ Rituximab in 188), 66 III generation regimen, 105 alkylators, 19 purine analogues, 9 other regimens, 19 rituximab alone. Doses of chemotherapy were reduced in 33% of pts. Among 280 pts with normal ALT at NHL diagnosis, 46 (16%) developed WHO hepatic toxicity ≥ grade 2; among 197 pts (42%) with abnormal ALT, 21 (11%) experienced ALT increase >3.5 times baseline value. Overall, severe liver toxicity developed in 67 pts (14%) (17% of aggressive NHL and 10% of indolent NHL, P=0.05). Use of Rituximab was not associated with severe liver toxicity (P=0.5). In DLBCL, R-CHOP and CHOP showed similar rates of severe hepatic toxicity (19% vs 17%, respectively; P=0.7), although R-CHOP determined higher HCV-RNA load: median increase of HCV-RNA during treatment was 3,356,300 IU/mL in R-CHOP group and 1,566,600 IU/mL in CHOP group (P=0.01). Maximum grade of liver toxicity was registered before 3rd cycle in 61% of pts treated with R-CHOP and in 30% of those treated with CHOP (P=0.008). Steroids were stopped or reduced in 100 pts (in 53 for liver toxicity). 91 pts did not complete planned therapy (22 for liver toxicity). Treatment administration was postponed at least one time in 147 pts (in 33 for liver toxicity). After a median F-UP of 2.7 years, 143 pts died (19 for liver failure). 5-yrs OS was 84% for indolent NHL and 70% for aggressive NHL. 5-yrs PFS was 38% for indolent NHL and 46% for aggressive NHL. In DLBCL, IPI and R-IPI were predictive of OS and PFS (P<0.001 for all). *Conclusions.* A significant percentage of pts with HCV-positive NHL, when treated with conventional (immuno)-chemotherapy, develop severe liver toxicity often leading to reduction of doses or interruption of treatment. In HCV-positive DLBCL, addition of rituximab to CHOP scheme does not increase hepatic toxicity; in this setting IPI and R-IPI retain prognostic value.

0281

CLINICAL FEATURES OF NON HODGKIN'S LYMPHOMAS ASSOCIATED WITH HEPATITIS C VIRUS INFECTION: STUDY OF THE 'REGISTRO LOMBARDO DEI LINFOMI HCV-POSITIVI' (LOMBARDY REGISTRY OF HCV-POSITIVE LYMPHOMAS, ITALY)

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Background. Hepatitis virus C (HCV) infection is a health problem all over the world. In Italy, particularly in the southern region, the infection is endemic. In Lombardia, a region of northern Italy with nearly ten millions of inhabitants, the prevalence of infected people is around 5%. In the last years, increasing evidence showed a role of HCV in the etiology of B-cell lymphomas. *Aims.* in January 2008 we launched the 'Registro Lombardo dei Linfomi HCV-positivi (Lombardy Registry of HCV-positive lymphomas, Italy), a prospective observational study, involving the Hematology Centres of "Rete Ematologica Lombarda"(REL) (Lombardia Hematology Network), with the aim to collect histological, clinical and therapeutic data of HCV-positive lymphomas in Lombardy region. This study was approved by the Regional Administration and by IRBs of participating institutions. All pts provided written informed consent. *Methods.* Since January 2008, we collected data of 140 consecutive patients (pts) with lymphoma and HCV infection diagnosed in the participating centres. *Results.* 51 were males and 89 females; median age at diagnosis was 67 years (range 32-90). Histotypes were as follows: 62 aggressive lymphoma (60 DLBCL), 40 marginal zone lymphomas (MZL) (16 splenic, 11 nodal, 13 extranodal of MALT), 14 follicular lymphoma, 3 lymphoplasmacytic lymphoma, 2 lymphocytic

lymphoma, 1 mantle cell lymphoma, 18 low-grade B-cell lymphoma NOS). Ann Arbor stage was III-IV in 82% of cases; 74 showed extranodal localizations (spleen in 29, skin in 15, salivary glands in 5, ocular adnexa in 5). ECOG score was >2 in 21. Detection of HCV positivity preceded lymphoma diagnosis in 98 pts, of whom 18 had cirrhosis; in the remaining 42 pts, the first detection of HCV-positivity and the diagnosis of lymphoma were concurrent. In 14 pts liver biopsy revealed localization of lymphoma (8 DLBCL, 3 MZL, 3 low-grade B-cell lymphoma NOS). At diagnosis of lymphoma, 110/122 (90%) pts showed a detectable HCV-RNA with prevalence of genotype 1b and 2; 4/130 pts were HbsAg positive, without active replication. AntiHBc antibodies were present in 36/106 (34%). Cryoglobulins were detected in 21 pts and a serum MC was found in 43 pts (29 IgM, 14 IgG). Cryoglobulinemia (P=0.01), serum MC (P=0.003) and autoimmunity (P=0.003) were statistically associated with indolent lymphomas. Fourteen pts with indolent lymphoma were treated with antiviral therapy only and 9 obtained regression of lymphoma. Data on chemotherapy were available for 94 pts (+ rituximab in 57). Nine pts experienced grade 3-4 WHO liver toxicity. 55 pts (67%) achieved CR, 27 (33%) PR. *Conclusions.* this prospective collection of data from a regional network confirms the association of HCV infection with diffuse large B-cell lymphoma and marginal zone lymphomas. Antiviral therapy is able to induce regression of indolent lymphoma in nearly two thirds of treated pts. These data allowed to acquire new understanding on the natural history of HCV-positive lymphomas through the integration of information among hematology centres from homogenous geographical area. It represents an epidemiological and clinical basis for studies aimed to interrupt the pathogenic cascade and to identify best treatment for HCV-driven lymphoproliferation.

0282

A PHASE II TRIAL TO INVESTIGATE THE LINK WITH INFECTIOUS AGENTS IN OCULAR ADNEXAL MARGINAL ZONE LYMPHOMA, ESPECIALLY WITH CHLAMYDIA SPECIES, AND THE ANTINEOPLASTIC EFFECTS OF DOXYCYCLINE (IELSG#27)

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Background. Marginal zone B-cell lymphoma of the ocular adnexae (OAMZL) is associated with Chlamydophila psittaci (Cp) infection. The prevalence of infection has always been assessed in retrospective series, and varies around the world from 0% to 75%. Treatment with doxycycline has been associated with lymphoma regression in half of patients; prolonged contact with infected animals may determine continuous reinfection and hinder therapeutic efficacy. *Aims.* To define the prevalence of chlamydial and other infections and the eradicating and antineoplastic effects of doxycycline in OAMZL patients. *Methods.* Patients with newly diagnosed lymphoproliferative disorders of the ocular adnexae of any type were registered in an international multicenter phase II trial: patients with limited-disease (stage IEA) OAMZL and a measurable lesion were registered in the part A of the trial, received doxycycline 100 mg bid daily for 21 days and were assessable for infections prevalence, bacterial eradication and tumor response; all the others entered into the part B, were treated following the best local practice (doxycycline was an option) and were assessable for infections prevalence and eradication. The trial included molecular assessment, with different PCR protocols, of the presence of several bacteria and viruses in lymphomatous tissue, conjunctival swab, and peripheral blood mononuclear cells (PBMC). Eradication and response after doxycycline were assessed by clinical examination, conjunctival swab, PBMC sampling, and MRI of the orbits at 3 and 12 months from treatment. The primary endpoint was response rate and duration after doxycycline; the secondary endpoints were bacterial eradication, identification and geographical distribution of infectious agents, and identification of molecular factors predicting response to doxycycline. *Results.* Since October 2006 to February 2010, 49 patients were enrolled (32 in part A, 17 in part B). Hystotypes were MZL in 43 patients, pseudotumor in three, follicular lymphoma in two, and diffuse large B-cell lymphoma in one. Chlamydial infections were assessed in tumor tissue from 33 OAMZL patients and 4 non-OAMZL: Cp was detected in 27 (82%) and 3 (75%) patients, respectively. All cases were negative for C. pneumoniae and C. trachomatis. Among the 27 Cp-positive OAMZL, the microorganism was detected in 100% (n=27) of conjunctival swabs and in 78% (n=21) of PBMC samples. Swabs and PBMC were negative in Cp-negative lymphomas. Thirty-nine patients received doxycycline; bacterial eradication was assessed in 30 patients with Cp-positive swab (n=6) or Cp-positive swab and PBMC (n=24). After 3 months from antibiotics, eradication was obtained in 9/30 (30%) swabs and in 7/24 (29%) PBMC. Animal exposure (cats, birds and dogs) during treatment was not correlated with Cp eradication since 3 of the 9 eradicated patients and 6 of the 21 patients with persistent infection (Fisher exact test; P=0.99) maintained household animals during and after doxycycline treatment. Tumor response analyses will require a longer follow-up. *Conclusions.* This is the first prospective trial demonstrating that Cp is common in newly diagnosed OAMZL. Cp eradication with doxycycline failed in two thirds of patients, a feature not explained simply by animal exposure. This finding deserves to be better investigated since its potential negative effect on lymphoma regression.

0283

A PROSPECTIVE STUDY OF PREVALENCE, IMMUNOLOGIC RESPONSES AND OUTCOME OF EBV-ASSOCIATED LYMPHOMA IN THAI

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Background. Epstein-Barr virus (EBV) is an oncogenic virus associated with various types of lymphoma. The prevalence of EBV-associated lymphoma varies with geographic location. The role of EBV and immunologic responses to the virus and outcome in Thai patients remain unknown. Therefore, we prospectively study the prevalence, immunologic responses and outcome of EBV-associated lymphoma in Thai patients.

Table.

	1-yr Overall Survival	*P* value
B-cell NHL		
EBV-associated B-cell NHL	50%	0.018
Non-EBV-associated B-cell NHL	85.4%	
T/NK-cell NHL		
EBV-associated T/NK-cell NHL	25%	0.018
Non-EBV-associated T/NK-cell NHL	100%	
HL		
EBV-associated HL	100%	0.221
Non-EBV-associated HL	50%	

Methods. A prospective study of all newly diagnosed lymphoma in immunocompetent patients was done at King Chulalongkorn Memorial Hospital between January and October 2009. Patients who were immunocompromised or HIV-positive were excluded. *in situ* hybridization for EBV-encoded small RNA (EBER) was performed in all paraffin-embedded primary tissues; positive reaction was defined as EBER nuclear signal more than 5% of tumor cells. Blood samples were collected before starting treatment to measure cytokine (IFN-γ, IL-2) production from EBV-activated $CD8^+$ cytotoxic T lymphocytes (CTL) using enzyme-linked immunospot assay. The histopathology, EBER expression, CTL responses and patient outcome were analyzed. *Results.* There were 97 cases included in the study. EBV-associated lymphoma was detected in 5/76 (6.6%) of B-cell non-Hodgkin lymphoma (NHL) [4 diffuse large B-cell lymphoma (DLBCL), 1 Burkitt], 5/15 (33.3%) of T/NK-cell NHL [3 aggressive NK cell leukemia/lymphoma, 2 nasal-type NK/T-cell lymphoma], and 3/6 (50%) of Hodgkin lymphoma (HL) [2 mixed cellularity, 1 nodular sclerosis]. All EBV-associated T/NK-cell NHL and HL were strongly positive for EBER nuclear signal at 80-100% of tumor cells, in contrast to EBV-associated DLBCL, which had variable expression of EBER at 5-100% of tumor cells. All patients with diagnosis of aggressive NK cell leukemia/lymphoma and extranodal NK/T-cell lymphoma were EBER-positive. Among 37 patients with DLBCL, EBER was positive in 4/37 (10.8%) of cases. All EBV-associated DLBCL patients were older than 70 years old and had no CTL responses to EBV. Patients with EBV-associated DLBCL were significantly older than those with EBV-associated T/NK-cell NHL and HL (median age 75 vs. 51 vs. 43, P<0.05). None of the patients with EBV-associated HL had

CTL responses to EBV. Among 4 evaluable patients with EBV-associated T/NK-cell NHL, CTL responses to EBV were detected in 2/4 (50%) of cases. At a median follow-up of 9 months, the outcome of EBV-associated and non-EBV-associated lymphoma was summarized in the Table. *Conclusion.* Using EBER expression of more than 5% of tumor cells, our study revealed similar prevalence of EBV-associated B-cell NHL and HL but lower prevalence of EBV-associated T/NK-cell NHL, when compared to the reports from other Asian countries. Defective CTL responses to EBV might contribute to the development of lymphoma in most patients. The prognostic significance of EBV to the patient outcome needs to be confirmed with more patients and longer follow-up.

0284

CLINICAL CHARACTERISTICS, TREATMENT AND OUTCOME OF LATE POST-TRANSPLANTATION LYMPHOPROLIFERATIVE DISORDER AFTER KIDNEY TRANSPLANTATION: A MONOCENTRIC ANALYSIS OF 29 PATIENTS

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Background. Adult post-transplantation lymphoproliferative disorders (PTLD) are rare and severe complications of solid organ transplantation (SOT). Early and late-onset PTLD occur during or after the first year post-SOT respectively. Compared to early-onset PTLD which usually respond to immunosuppression withdrawal, treatment of late-onset PTLD is less defined. *Aims.* To analyze the outcome of late PTLD after kidney transplantation diagnosed after 2000 in our institution. *Methods.* Clinical and histological data for 29 patients followed at our center who developed late PTLD between 1999 and 2009 after kidney transplantation were retrospectively analyzed. *Results.* 29 patients were diagnosed with a PTLD at our institution. The median interval between SOT and PTLD was 134.6 months (range 29 to 338 months). 25/29 patients (86%) had extranodal disease. Digestive tract was involved in 27.5% of patients (8/29), pulmonary localization was found in 13.7% (4/29) and central nervous system was involved in 13.7% of patients (4/29). Bone marrow involvement was found in 24.1% of patients (7/29). 19/29 patients were Ann Arbor stage III/IV (65.5%). 7% of patients had polymorphic PTLD (2/29 patients), 10% had classical Hodgkin lymphoma-type PTLD (3/29) and 83% had monomorphic PTLD (24/29). 100% of polymorphic- and 33% of monomorphic- PTLD were EBV-positive. 50% of the latter were EBV-negative and EBV status was unknown in 16.6%. 80% of monomorphic PTLD were B-cell lymphoma (19/24) and 20% were T-cell lymphomas (5/24). The majority of B-cell lymphomas were diffuse large B-cell lymphoma (89%, 17/19), 1 patient had a Burkitt lymphoma and 1 patient had an extranodal marginal zone lymphoma. All but three patients had a reduction of immune suppression: calcineurin inhibitors and antimetabolite were stopped and corticosteroids were increased. 19/29 patients (65%) received rituximab-based treatment. 4 patients (13.8%) received rituximab only and 15 (51.7%) received rituximab combined to chemotherapy. Among patients treated without rituximab, one had complete remission with decreased immune suppression, one received corticosteroid only and 8 (27%) received chemotherapy. During 28 months median follow-up (range 1 to 120 months), complete remission was obtained in 72% of cases (21/29). 2-years overall survival was 72%, 2-years progression free survival 68%. 1 patient was diagnosed for a myeloid acute leukemia 27 months after lymphoma treatment and rapidly died. Complete remission was obtained in 74% (14/19) of patients who received rituximab during treatment and in 70% of patients (7/10) who did not received rituximab. 2-year overall survival and progression-free survival were respectively 74% and 74% for patients who had rituximab, 60% and 40% for those who did not received rituximab. Kidney function was preserved in 93% cases. 2-years mean creatinine value was 1,86mg/dL (SD=0.83 mg/dL). One patient had to resume dialysis and one patient had second kidney transplantation. *Discussion/Conclusion.* This monocentric, retrospective analysis suggests improved overall survival and progression-free survival for patients who received rituximab for treatment of late PTLD after kidney transplantation. Analysis to determine impact of changes in immune suppression protocols on risk factors and outcomes of PTLD based on the comparison with a previous cohort form our center (Mamzer-Bruneel *et al*. J Clin Oncol 2000) is ongoing.

0285

HEPATITIS B REACTIVATION IN PATIENTS WITH NON HODGKIN LYMPHOMA CD20+ UNDERGOING CHEMOTHERAPY

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Background. Reactivation of HBV infection is a well-recognized complication in infected patients who undergo cytotoxic chemotherapy for cancer. The highest incidence of reactivation was reported in patients with non-Hodgkin's lymphoma (NHL) and hematopoietic stem cell transplantation. Several case reports demonstrated that severe hepatitis due to HBV reactivation after rituximab administration occurred both in hepatitis B surface antigen(HBsAg)-positive and HBsAg-negative patients. However, systematic evaluation of the relationship between HBV reactivation and rituximab is still limited. Thus, we conducted a study to investigate the relationship between rituximab-based therapy and HBV reactivation in 230 CD20-positive NHL patients at our institution. *Patients and Methods.* In our Unit, 230 CD20-positive NHL patients all newly diagnosed underwent measurement of HBsAg, anti-HBs,anti-HBc,HBeAg and anti-HBe. Patients were monitored by liver function tests during and after therapy as follows: on day 1and day 14 of each cycle, every month for a year. *Results.* 106/230 patients were HBcAb-positive. 73/106 had an aggressive lymphoma, 36 had an indolent lymphoma. HBV reactivation was observed in one patient (2,3%) who had received chemotherapy including steroid and rituximab and in 3 patients(15,7%) who had received chemotherapy including regimen with only fludarabine without rituximab (Table 1). Immediate administration of lamivudine therapy after elevation of HBV DNA level was conducted, and this resulted in reduction of it and improvement of liver function test. *Conclusions.* Rituximab plus steroid-containing regimens may increase the risk of HBV reactivation in HBsAg-negative and HBcAb-positive lymphoma patients but more attention should be paid on treatment with only fludarabine More ambitious prospective studies are required to establish clinically useful or cost-effective follow-up methods for control of HBV reactivation in lymphoma patients with occult HBV infection.

Table 1. Results of the analyses of 106 patients HBsAg Negative /anti- HBc positive.

Therapy	*Isolated anti-HBc patients*	*N°reactivation*	*% reactivation*
R-CHO	44	1	2,3
CHOP	29	0	0
R-Fluda	17	0	0
Fluda	9	3	15,7

0286

COMBINATION OF RITUXIMAB WITH CHLORAMBUCIL AS FIRST LINE TREATMENT IN PATIENTS WITH MANTLE CELL LYMPHOMA: A HIGHLY EFFECTIVE REGIMEN

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Background. Optimal treatment approach for mantle cell lymphoma (MCL) patients is not well defined. *Aim.* To present our experience on Rituximab-chlorambucil combination, a totally different therapeutic strategy of low toxicity which has been recently adopted by us, as first line treatment in patients with MCL. *Patients and Methods.* From June 2004 to August 2008 twenty consecutive patients with MCL (14 males and 6 females), except the ones with blastic variant of the disease, received the combination of Rituximab and chlorambucil as first line treatment. Patients' characteristics are shown on Table 1. Initial staging was based on the Ann Arbor system. All patients underwent bone mar-

row aspiration and biopsy as well as lower gastrointestinal tract endoscopy. IgVH analysis and FISH for the detection of t(11;14) translocation was performed at diagnosis and at reevaluation using standard techniques Response was defined using standard criteria. Further on molecular response was defined as the absence of detection of IgVH rearrangement and/or t(11;14) translocation in bone marrow/blood. Treatment schedule included an induction and a maintenance phase. At induction patients received Rituximab at a dose of 375 mg/m^2 at day 1 of each 28 day cycle. Chlorambucil was given at a fixed dose of 10mg/day for 10 consecutive days of each cycle starting the day after Rituximab administration. Patients received 8 cycles of the above combination. Thereafter chlorambucil was administered alone for 4 additional cycles. At maintenance patients in complete or partial remission after the end of induction phase received rituximab at a dose of 375 mg/m^2 every two months for 1 year. Response evaluation was assessed at the end of 8th and 12th chlorambucil cycle and at the end of maintenance phase. *Results.* After the 8th cycle 13 patients presented CR (65%) and 7 PR (35%) while after the end of induction treatment (12th cycle), CR was documented in 18 patients (90%), 1 PR (5%) and one disease progression. Overall response rate was 95%. Of the 13/18 patients in CR with demonstrable IgVH rearrangement in bone marrow at diagnosis, 12 were found to be in molecular remission. The median follow up time of our patients was 22 months (range 14-55). The 2-year progression free survival was 82%. At last follow up 16/18 pts are still in CR. Two pts in CR (11%) experienced disease relapse twenty and fourteen months after the end of induction therapy respectively. Patient in PR had stable disease until last follow up while the one with progressive disease received a fludarabine based regimen and regression of his disease was noted. In 4 younger patients in whom a molecular remission was achieved stem cell collection was performed. Treatment was well tolerated without serious haematological toxicities. *Conclusions.* The combination of Rituximab plus chlorambucil as first line treatment in MCL patients is effective inducing high response rates. Molecular remission is achievable and stem cell collection is feasible. Tolerance of treatment is excellent and all patients enjoy a high quality of life.

Table 1. Characteristics of 20 MCL patients.

Characteristic	*Patients*	
Age-median (range): 64 (32-84)	#	%
Male/Female	14/6	70/30
B-symptoms	2	10
Generalized lymphadenopathy	10	50
Bone marrow involvement	17	85
Blood involvement	11	55
Extranodal sides	6	30
Splenomegaly	5	25
Ann Arbor stage III/IV (advanced)	17	85
IPI score: 0-1/ 2/ 3/ 4-5	6/12/1/1	30/60/5/5
MIPI risk: Low/ Intermediate/High	13/6/1	65/30/5
Hb‹12 gr/dl	4	23
LDH›UNL	2	11
Ki-67 Low (≤10%) Intermediate (11-29%) High (≥30%)	2/12 6/12 4/12	16 50 33

0287

THE ROLE OF MANTLE CELL INTERNATIONAL PROGNOSTIC INDEX (MIPI) AS SURVIVAL PREDICTOR IN MANTLE CELL LYMPHOMA (MCL) PATIENTS TREATED WITH RITUXIMAB AND CHEMOTHERAPY

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Background. Clinical course of MCL is unfavourable, with a pattern of continuous relapses. The recent MIPI, a clinical and biological score, defined performance-status, age, LDH and leucocyte counts as predictors of MCL outcome. Ki-67 as cell proliferation index was evaluated in biological-MIPI (MIPI-b). *Aims.* The study was a retrospective study. Primary endpoint was to tested MIPI on a retrospective group of MCL patients treated with Rituximab-chemotherapy; secondary endpoints were: to evaluate the feasibility of MIPI-b on a retrospective population and to quantify the predictive discrimination of IPI, MIPI, MIPI-b on the outcome of MCL in the Rituximab era. *Methods.* Between 1999 and 2009, 136 MCL >18 years at diagnosis consecutively treated in five Italian institutions entered into the study. Histology was centrally reviewed and Ki-67 evaluation was performed. Overall Survival (OS) and failure-free survival (FFS) curves were estimated both overall and stratified by MIPI, MIPI-b and IPI score. Differences between curves were tested using the 2-tailed log-rank test. In order to quantify the predictive discrimination of MIPI, MIPI-b and IPI scores, a Cox's model analysis and univariate logistic models (with death and failure event as binary outcomes) were fitted and the area under the receiver operating characteristic (ROC) curves (c-index) was estimated in a subgroup of 84 patients that fulfilled MIPI, MIPI-b and IPI scores. *Results.* Clinical characteristics were: median age 62 (37-84) years, 78% stage IV, 73% with bone marrow involvement, 15% with blastoid variant; median leucocyte counts at diagnosis was 7.53×10^3 (2.38-175). First-line treatments were: Rituximab with high-dose chemotherapy in 35%, Rituximab-Fludarabine based chemotherapy in 16%, Rituximab-CHOP in 37% and other Rituximab containing regimens in 12%. Ki-67 evaluation was performed in 93 patients. Patients at high-risk (HR) were 43 patients (32%) according to MIPI, 16 (12%) according to MIPI-b and 47 (35%) to IPI. With a median follow-up of 28 months, 2-year OS was 77% (95% CI:68%-84%) and 2-year FFS was 60% (95%CI: 50%-69%). Two-year OS according to MIPI and IPI were shown in Figure 1. In the subgroup of 84 patients that fulfilled MIPI, MIPI-b and IP scores an univariate logistic model and a Cox's model analysis were fitted. The c-index and Cox-index for death event were 73% and 77% for MIPI, 72% and 73% for MIPI-b, 69% and 65% for IPI respectively; the c-index and Cox-index for failure event were 66% and 72% for MIPI, 66% and 69% for MIPI-b, 66% and 64% for IPI respectively. *Conclusions.* MIPI score was confirmed as a good predictor of death event in MCL retrospective patients treated with Rituximab-chemotherapy regimens. MIPI score should predict outcome better than MIPI-b and IPI in a retrospective analysis. MIPI and MIPI-b score superiority on standard IPI should be demonstrated in prospective trials. New therapeutic strategies are warranted to improve the outcome of MCL namely in MIPI-HR group.

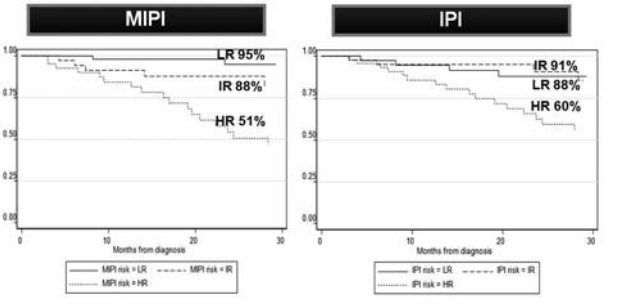

Figure 1.

0288

SALVAGE TREATMENT WITH LENALIDOMIDE AND DEXAMETHASONE IN PATIENTS WITH RELAPSED REFRACTORY MANTLE CELL LYMPHOMA

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Background. Previous *in vivo* and *in vitro* studies have highlighted the activity of Lenalidomide (Len) in patients with relapsed or refractory mantle cell lymphoma (MCL), achieving a 53% overall response rate (ORR), which included 20% complete responses (CR) (Habermann et al. Br J Haematol. 2009) and the possible synergistic anti-proliferative effect of Dexamethasone (Dex). *Aim.* On this basis we initiated a prospective, multicenter, phase II study, to evaluate safety and efficacy of Len Dex combination for adult patients with MCL. *Methods.* Patients had to have ≥1 prior treatment regimen, and were either not eligible for, or had relapsed after, more intensive treatments including stem cell transplantation (SCT). During the induction phase (month 1 to 3), patients received Len 25 mg/day on days 1 to 21 and Dex 40 mg/day on days 1, 8, 15, 22 of a 28-day cycle (Len Dex). Patients who achieved a partial response (PR) or stable disease (SD) at the end of the induction phase continued to the consolidation phase, which consisted of treatment with Len Dex until disease progression, unacceptable toxicity, for a maximum of 12 months. Patients with a CR at the end of the induction phase, or those who achieved a CR during consolidation, received an additional 3 courses of Len Dex. The primary objective was to evaluate the ORR and CRR. *Results.* Between July 2008 and July 2009, 33 patients were enrolled on this study. Patients' median age is 68 years (range 51-80); 30 have the classic histology while 3 patients have the blastoid variant; 9 patients previously received two lines of therapy, 9 patients had three lines and 12 patients had >3 prior lines (median 3; range 1-7). Twelve patients previously underwent an autologous SCT and 7 received prior therapy with Bortezomib. At present, all patients are valuable for response to the induction phase of the study, 13 discontinued therapy prematurely, 8 completed the therapeutic program, 12 are still on therapy. Twenty-one patients responded to Len Dex (64% OR), including 4 CRs (12%); 1 patient (3%) has SD and 11 patients (33%) either had not responded or had progressed while on study. None of the CR patients had subsequent progression; among the 17 patients in PR, during consolidation therapy, 2 had subsequent progression, 2 achieved CR and 13 maintained the PR status. Most common Grade 3-4 adverse events scale of 1 to 5 were hematologic and included neutropenia (42%), thrombocytopenia (15%) and anemia (12%). Other events included 4 patients (12%) with grade 3-4 neutropenic fever and 3 patients (9%) with grade 3 bacterial pneumonia. Grade 3-4 hypotension and dispnea developed in 1 patient each, and none of the patients developed thrombo-embolic or neuropathic complications. *Conclusions.* Initial results from this study confirm the high therapeutic activity of Len in patients with relapsed and refractory MCL with a favourable safety profile; the addition of Dex does not appear to substantially improve the activity of Len in this subset but need further investigation.

Lymphoma - Clinical 2

0289

18F-FLUORODEOXYGLUCOSE POSITRON EMISSION TOMOGRAPHY (FDG PET) RESPONSE PREDICTS SURVIVAL IN PRIMARY MEDIASTINAL LARGE B-CELL LYMPHOMA

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Background. Primary mediastinal B-cell lymphoma (PMBCL) is a distinct clinicopathological variant of large B-cell lymphoma. The optimal treatment is unknown, with some studies demonstrating favorable outcome compared to other types of diffuse large B-cell lymphoma. Role of 18F-fluorodeoxyglucose (FDG) positron emission tomography (PET) in this entity is not well reported. *Aims.* In this retrospective study, we evaluated the primary presentation, clinical characteristics, treatment outcome and impact of FDG PET on PMBCL management at our institution. *Methods.* All patients with PMBCL diagnosed and managed during 1995-2008 at our institution were identified from Oncology Data Unit. Patient's characteristics, prognostic factors, details of treatment and outcome were reviewed. *Results.* Forty-seven patients were identified. Median age was 30 years (range 18-66). There were 26 (55%) females and 21 (45%) males. Forty-two patients (89%) had stage I-II disease. Seventeen patients (36%) had ECOG performance status of >2. Bulky disease in 37 (79%) patients. All patients received CHOP (with Rituximab in nine patients) chemotherapy followed by radiation therapy in 42 (89%) patients. Complete response rate + complete response unconfirmed was 76.6%. Median follow-up was 50.5 months and median overall survival (OS) and event free survival (EFS) were not reached. Univariate analysis showed female gender, early stages and response to treatment as good prognostic factors with higher OS and EFS, while response to planned treatment was the only factor that had impact on OS in multivariate analysis in all 47 patients. Twenty-five patients had FDG PET scan after chemotherapy. On Univariate analysis FDG PET negative patients showed significant improvement in OS and EFS compared to patients who were FDG PET positive; median OS and EFS were not reached in FDG PET negative patients and they were 22.2 and 5.9 months respectively in FDG PET positive patients (P=0.002 & 0.004 respectively). On multivariate analysis, female gender (P=0.046) was the only significant factor among metabolic response (P=0.099), CT response (P=0.518), and age (P=0.231). Regardless of IPI score, patients with metabolic CR on FDG PET had an excellent OS (P=.0005) and EFS (P=0.0056) Figure attached (OS). *Conclusions.* In conclusion, Female gender carries favorable prognosis in PMBCL as reported previously in the literature. More importantly, despite having small number of patients with FDG PET, our data supports that achieving metabolic CR on FDG PET leads to significant prolongation of OS & EFS. Metabolically negative FDG PET is stronger predictor of OS and EFS compared to IPI in PMBCL.

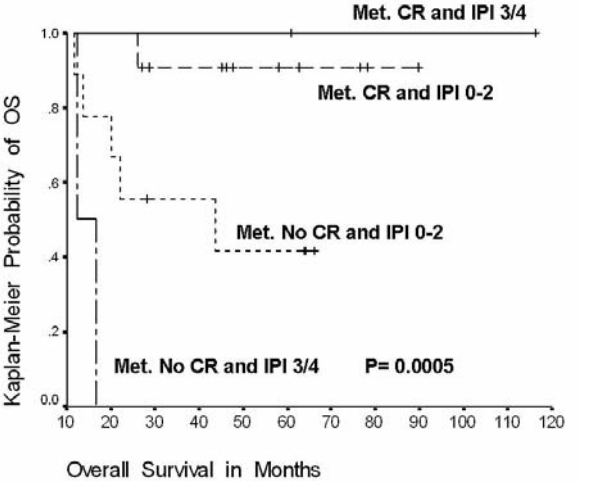

Figure.

0290

PROGNOSTIC FACTORS FOR THE OUTCOME OF PATIENTS WITH PRIMARY MEDIASTINAL LARGE B-CELL LYMPHOMA (PMLBCL) UNDER CHEMOTHERAPY WITH RITUXIMAB-CHOP (R-CHOP) WITH OR WITHOUT RADIOTHERAP

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Background. Prognostic factors have not been extensively studied in PMLBCL, obviously due to its rarity. Based on data extracted prior to the Rituximab era, the International Prognostic Index (IPI) appears to retain its prognostic significance in several but not all studies. R-CHOP provides very good results in PMLBCL, minimizing failure rates. In historical control comparisons, Rituximab-chemotherapy combinations appear to be much more efficient than the same chemotherapy alone. Thus the applicability of various prognostic factors needs to be reevaluated in the Rituximab era. *Aims.* The identification of prognostic factors for the outcome of patients with PMLBCL treated with RCHOP±Radiotherapy (RT). *Patients and Methods.* 57 patients with PMLBCL aged ≤60 years old were treated in 7 centers in Greece with RCHOP±RT (usually 6-8 cycles). The following potential prognostic factors were evaluated: Gender (female 61%), B-symptoms (39%), stage III/IV (16%), extranodal involvement (32%), performance status (PS) ≥2 (17%), LDH levels (83%), anemia (43%), leukocytosis ≥10×10^9/L (20%), lymphocytopenia <1×10^9/L (33%), ESR ≥30 mm/h (65%), albumin <4 g/dL (36%), bulky disease (≥10 cm; 60%), age-adjusted IPI (aaIPI; ≥2 in 22%). *Results.* The median age of the patients was 30 years (17-59) and the median follow-up of currently alive patients was 40 months. All 11 failures occurred within 17 months from diagnosis: There were 5 early progressions and 1 late progression (relapse), while 5 patients were considered as failures, because they were forwarded to salvage therapy or additional procedures without prior overt progression (usually due to persistent PET findings). The 3-year failure free survival (FFS) was 80±5%. With only 5 deaths recorded, the 3-year overall survival (OS) was 91%. The aaIPI identified a minority of patients (those with aaIPI ≥2; 22% of total) with a 3-year FFS of 49% vs. 90% for those with aaIPI 0-1 (P=0.001). Among its individual components, stage and PS were significant predictors of FFS (P<0.05), while LDH was of borderline significance (P=0.10). Among other potential prognostic gactors examined, only ESR ≥30 (P=0.05), bulky disease (P=0.18) and B-symptoms (P=0.19) were marginally correlated with inferior FFS. Overall survival was not predictable by any of the examined prognostic factors: 3-year OS was 92% vs. 81% for patients with aaIPI 0-1 and ≥2 respectively. *Conclusions.* RCHOP±RT provided very good results in PMLBCL with long-term FFS of 80%. Only the aaIPI was predictive of the outcome, defining a rather small subgroup of patients with a ~50% probability of failure. However, OS was not poor even in this subgroup. The low failure rate and the rarity of the disease make the cooperation between groups necessary in order to accurately define prognostic factors in PMLBCL. Elevated ESR, bulky disease and B-symptoms might add to the prediction achieved by aaIPI, but this should be further evaluated in multivariate analysis.

0291

HIV-POSITIVE PATIENTS DIAGNOSED WITH BURKITT LYMPHOMA DID NOT SHOW DIFFERENCES IN TERMS OF RESPONSE AND OUTCOME AS COMPARED WITH A COHORT OF HIV-NEGATIVE PATIENTS DIAGNOSED AT THE SAME PERIOD OF TIME

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Background. Burkitt lymphoma (BL) is a highly aggressive non Hodgkin lymphoma often presenting in extranodal sites that accounts for 1-2% of all adult lymphomas in Western countries. HIV-positive patients more frequently develop BL. *Aim.* To analyze the clinical features and outcome of HIV-positive patients with BL and to compare them with the cohort of HIV-negative patients diagnosed with BL in the same period of time. *Methods.* Between 1994 and 2009, 47 patients (21 HIV-positive; 26 HIV-negative) were diagnosed with BL in a single institution, according to the WHO classification criteria. All HIV-positive patients received HAART therapy. Main clinical and evolutive variables were analyzed, including IPI. The treatment included intensive regimens in 23 cases. Rituximab was added after 2002. *Results.* Initial characteristics according the HIV-status are detailed in the Table. The median CD4 in HIV this population at the time of diagnose was 285 cells/µL (33-540). CR rate, early death rate, progression-free survival (PFS) and overall survival (OS) according to the HIV-status are listed in the table. As it can be observed, no differences were found between the two groups. Patients receiving intensive regimens showed higher OS than those with no intensive therapies (5-year OS 81 vs. 52%, respectively; P=0.009). No differences in the outcome of the patients were observed according to the rituximab treatment in either HIV-positive or HIV-negative patients. 18 patients have died after a median follow-up of 4.4 years. Causes of death included progression (4 cases), tumour lysis (1), CMV encephalitis (1) and prostate neoplasm (1) in HIV-positive patients, and progression (5), sepsis (4), liver cirrhosis (1), and complications of allogeneic transplantation (1) in HIV-negative patients. *Conclusions.* In the current series of patients diagnosed with BL the HIV-status was not relevant to predict the outcome of the patients.

Table.

Number of Patients	*HIV positive* N=21	*HIV negative* N=26
Age (median)	39 (17-59)	38 (17-9)
Gender (Male/female)	17 / 4	18 / 8
Performance status ECOG>2	48%	50%
B symptoms	61%	58%
High serum LDH (%)	76%	73%
Advance Stage (IV)	90%	62%
Extranodal involvement ≥2 sites	48%	23%
IPI Low risk Intermediate risk High risk	 19% 52% 29%	 35% 39% 27%
Treatment Rituximab regimens High Intensive regimens	 58% 52%	 30% 46%
CR rate All cases Intensive treatment	 15 (71%) 11 (92%)	 17 (64%) 9 (90%)
Early death (<4 months)	4 (19%)	5 (19%)
5-year PFS	66%	52%
5-year OS	71%	60%

0292

PHASE 1/2A STUDY OF NAVITOCLAX (ABT-263) IN RELAPSED OR REFRACTORY LYMPHOID MALIGNANCIES

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Background. Bcl-2 family members are associated with tumor initiation and drug resistance, and are compelling therapeutic targets. Navitoclax is a novel, orally bioavailable, small molecule BH3 mimetic that binds (Ki ≤1 nM) to Bcl-2, Bcl-xL, and Bcl-w with high affinity. Navitoclax displays potent preclinical cytotoxicity (EC50 ≤1 µM) against human tumor cell lines (small cell lung carcinomas and T and B lymphoid malignancies) that express Bcl-2. Preclinical toxicities are mechanism-based, with Bcl-2, Bcl-xL and Bcl-w mediating effects on lymphocytes, platelet survival and spermatogenesis, respectively. *Aims.* The aims of this study were to assess safety/pharmacokinetics and maximum tolerated dose of ABT-263. *Methods.* Phase 1/2a single-agent international study employing a modified Fibonacci 3+3 of navitoclax in relapsed/refractory lymphoid malignancies. Subjects were dosed on

Days (d) 1-14 of a 21-d dosing cycle with 10-440 mg navitoclax, or continuous 21/21-d administration (21-d cycle) with 200-425 mg navitoclax following a 150 mg lead-in dose. Tumor responses were evaluated using the IWG criteria and NCI-WG criteria (for CLL and SLL subjects). *Results.* To date, 55 subjects (38 on a 14/21-d and 17 on a 21/21-d dosing schedule) have received navitoclax. The median subjects age was 59 y (range, 20-81). Informed consent was obtained. The PK profile of navitoclax on the 14/21-d schedule was linear and dose proportional from 10-440 mg with a terminal t½ of approximately 17 h. Platelet nadirs were transient on the 14/21-d schedule and occurred on d 3-5. Data indicates a 1-week low dose lead-in at 150 mg navitoclax followed by a 21/21-d dosing schedule minimizes platelet reduction and cycle variability. Four subjects on the 14/21-d schedule had dose-limiting toxicities (DLT); 1 at 160 mg (bronchitis), 2 at 315 mg (elevated ALT and Grade 4 Thrombocytopenia) and 1 at 440 mg (atrial fibrillation). Three subjects on the 21/21-d schedule had DLTs; 1 at 275 mg (Grade 4 thrombocytopenia) and 2 at 425 mg (Grade 3 ALT and Grade 3 GI bleed). The median progression-free survival was 88 d [95% CI: 46-171]. Of the 55 subjects enrolled, tumor regression >50% was observed in 9 subjects: 1 NK-T cell lymphoma had a 75% reduction, 1 follicular lymphoma had an unconfirmed partial response (PR) and 7 CLL/SLL had >50% reduction in lymphadenopathy; 4 additional CLL had >50% decrease in circulating lymphocytes. Of 51 subjects who discontinued therapy, 40 did so due to progressive disease, 6 due to AEs, and 5 withdrew consent. *Conclusions.* Navitoclax is well tolerated with a linear PK, with toxicity due to on-target effects and antitumor activity in subjects with relapsed or refractory lymphoid malignancies. Thrombocytopenia was predictable and manageable. One week 150 mg/d lead-in followed by continuous dosing minimizes platelet nadir and cycle variability. Based on the 21/21d continuous dosing data, the modified Fibonacci 3+3 model projection for MTD converged. To mitigate platelet nadirs and stabilize cycle variability, the phase 2 recommended dosing regimen for navitoclax is 150 mg 7-d lead-in followed by 325 mg/d continuous dosing.

0293

EFFICACY AND SAFETY OF RITUXIMAB, DEXAMETHASONE, CYTARABINE AND OXALIPLATIN (R-DHAX) IN ELDERLY PATIENTS WITH RELAPSED/REFRACTORY B CELL NON HODGKIN'S LYMPHOMA

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Background. Salvage therapy for elderly patients with refractory/relapsed B cell non-Hodgkin's lymphoma (NHL) is based on polychemotherapy. Several salvage regimens are based on platinum, such as R-DHAP (rituximab, dexamethasone, cytarabine and cisplatin). This regimen is usually efficient in elderly patients but produce severe myelosuppression, peripheral neuropathy, and frequent renal toxicities that often require discontinuation of the treatment. Oxaliplatin is a third generation platinum drug without renal toxicity in contrast to cisplatin. *Aim.* To assess efficacy and safety of substituting cisplatin with oxaliplatin in R-DHAP regimen. *Methods.* We retrospectively analyzed a series of 48 elderly patients with refractory/relapsed B cell NHL treated with R-DHAX regimen (rituximab 375 mg/m^2 day 1, dexamethasone 40 mg days 1-4, oxaliplatin 100 mg/m^2 day 1, cytarabine 2000 mg/m^2/every 12 hours day 2, every 21 days). Analysis was done in patients 60 to 70 years (n=30) and older than 70 years (n=18). *Results.* The most frequent histological subtypes were diffuse large B cell lymphoma (n=27) and follicular lymphoma (n=8). Eleven patients (23%) were rituximab-naive at time of R-DHAX. Performance status was good (0-1) for 40 patients (83%). Twenty four patients (50%) had an elevated LDH level. Before R-DHAX, renal function was normal in 33 patients (69%). After Cockroft calculation renal insufficiency (RI) was moderate in 14 patients (29%) and severe in 1 patient (2%). For 21 patients (44%) R-DHAX was the second line of treatment. The median number of cycles received was 3 (range 1-7). Seven patients underwent subsequently autologous stem cell transplantation. Full dose-therapy was delivered in 50% of the patients aged between 60-70 and in 28% of the patients aged 70 and above. Doses were diminished at baseline in 13 patients and during the treatment in 9 patients. Toxicities of R-DHAX are summarized in Table 1. Grade III/IV toxicities were mainly hematological, including anemia (n=7, 15%), neutropenia (n=20, 42%), and thrombocytopenia (n=24, 50%). Grade III/IV neurological peripheral toxicities were observed in 3 cases (motor neuropathy n= 3, sensitive neuropathy n=1). Those 3 patients had prior exposure to vincristin, all of them had complete recovery at discontinuation of R-DHAX. Neither renal toxicities nor degradation of previous RI were observed. Dose adjustments were made at baseline for 16 patients (previous cisplatin exposure or prevention of toxicity) and during treatment for 12 patients (hematotoxicity n=5, neurotoxicity n=6, poor performance status n=1). The overall response rate was 73% (CR + CRu n=17) for patients younger than 70 years and 72% (CR + CRu n=10) for the patients older than 70 years. The median follow-up for living patients was 27.5 months months. The two-year probability rates of OS (overall survival) and of PFS (progression free survival) were 66% and 36% respectively. Summary/*Conclusions.* R-DHAX is an efficient salvage therapy in the context of relapsed/refractory B cell lymphoma in elderly patients, and its lack of renal toxicity makes it an attractive regimen that may be used in a large group of patients. Hematotoxicity was acceptable and did not result in unexpected complications when proper dosages were administered.

Table.

	60-70 years n= 30	> 70 years n= 18
	%	%
Anemia (Hb <8 g/dL)		
- Grade III/IV	10	22
- Patients who received ESA	67	44
- Grade III/IV with EPO	7	0
Neutropenia (Neutrophils < 1 G/L)		
- Grade III/IV	33	56
- Patients who received G-CSF	77	72
- Grade III/IV with G-CSF	20	33
- Febrile neutropenia requiring hospitalization, n	23	17
Thrombocytopenia (Plt < 50 G/L)		
- Grade III/IV	49	50
Grade III/ IV peripheral neuropathy	2	1
Induced Renal Insufficiency	0	0
Grade III/ IV digestive toxicity	0	0
Auditory toxicity	0	0
Grade III kerato-conjunctivitis	1	0

ESA: Erythropoiesis stimulating agents

0294

BENDAMUSTINE-R2ITUXIMAB COMBINATION TREATMENT IN RELAPSED NHL: COMBINED EXPERIENCE FROM TWO PHASE 2 TRIALS AND EXPLORATION OF RESPONSE PREDICTORS

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Background. Bendamustine plus rituximab (BR) is highly effective in previously treated patients with non-Hodgkin's lymphoma (NHL). Previous studies that individually evaluated the impact of BR used small sample sizes (Rummel *et al*, 2005; Robinson *et al*, 2008) without characterizing factors that influence outcomes. *Aims.* The aims of this study were to estimate the response rate and progression-free survival (PFS) based on updated data from both samples combined and to explore the predictors associated with complete response (CR) and PFS. *Methods.* Overall, 129 patients (median age, 62 years [range, 40-84], 61% male) were included in the combined analysis. Histologies included follicular (49%), mantle cell (22%), lymphoplasmacytoid (15%), small lymphocytic (8%), and marginal zone lymphoma (6%). Baseline characteristics were similar in the two studies, except that more patients in the Rummel study had stage III/IV disease and a WHO performance status ≥2. Patients were treated with bendamustine 90 mg/m^2 (30-minute intravenous infusion) on days 1 and 2 plus rituximab 375 mg/m^2 on day 1 for 4 to 6 cycles. All patients also received a single dose of rituximab 1 week before the first cycle and 4 weeks after the last cycle. We explored factors associated with (a) CR, using logistic regression modeling and (b) PFS, using Cox proportional hazard modeling. Age, gender, histological type, bone marrow involvement, spleen involvement, number of prior treatment regimens, and hematologic toxicities were reported in

a similar format across both studies. *Results.* The overall response rate (ORR) of partial response (PR) or better for BR was 91% (95% confidence interval [CI], 86%-97%), including 57% CR and 34% PR. Median follow-up time was 31.4 months, and median PFS for the combined population was 29 months (95% CI, 25-41). In the logistic regression analysis, male gender and stage IV disease were significantly associated with an inferior CR. Bone marrow involvement and spleen involvement were not significantly associated with CR. None of the predefined factors were significantly associated with PFS. Toxicity was mostly hematologic; no grade 3 or 4 toxicity was reported in the Rummel et al study population, as compared to 36% of the patients in the Robinson et al study. *Conclusions.* Pooling the data from 2 studies allowed the estimation of response rate and PFS with greater precision and facilitated the evaluation of predictors of progression in this setting. BR is a highly active regimen in previously treated NHL, demonstrating an ORR >90%. However, men and patients with stage IV disease were less likely to achieve CR. The lack of association between patient and disease characteristics and PFS during BR treatment suggests that BR is effective in inducing a response and slowing progression without strong dependence on age, histology, number of prior treatment regimens, and baseline bone marrow or spleen involvement.

0295

ANALYSIS OF A PHASE 2 STUDY OF LENALIDOMIDE AND RITUXIMAB IN RELAPSED OR REFRACTORY NON-HODGKIN'S LYMPHOMA

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Background. Despite an increase in our understanding of the pathophysiology of indolent non-Hodgkin's lymphoma (NHL) and the recent advances in early-line treatment options, the optimal treatment for patients with relapsed/refractory disease has not been determined. Rituximab has demonstrated clinical benefit in combination with chemotherapy for indolent NHL, and rituximab-based chemotherapy combinations are used both in first-line and salvage therapy. The immunomodulatory agent lenalidomide has demonstrated clinical activity in the treatment of B-cell hematological malignancies. In a recently published study, lenalidomide monotherapy resulted in a >16.5-month response duration in 43 patients with relapsed/refractory indolent NHL (Witzig *et al*. JCO 2009). In lymphoma cells and in animal models, the combination of lenalidomide and rituximab (R2) has shown improved clinical activity relative to treatment with either agent alone. *Aims.* To determine the clinical benefit and safety profile of R2 combination therapy in patients with relapsed/refractory indolent NHL (off-label). *Methods.* Patients with relapsed/refractory indolent NHL with measurable disease, ≥1 prior therapy, and ECOG performance status score ≤2 provided informed consent and were enrolled. Oral lenalidomide 25 mg/day was given on days 1-21 of a 28-day cycle, and was continued until disease progression. After 2 of 4 patients developed grade 3 tumor lysis syndrome (TLS), the protocol was amended to reduce the starting dose to 20 mg/day, and prophylaxis with allopurinol was initiated. Rituximab 375 mg/m^2 IV was administered on day 15 of cycle 1, and repeated weekly for a total of 4 doses. The overall response rate (ORR) was the primary endpoint, and secondary endpoints included response duration, overall survival, progression-free survival (PFS), and safety. *Results.* 16 patients were enrolled on study and are evaluable for response assessment. The median age was 60 years (50-91), and patients had received a median of 3 prior therapies (1-11). Ten patients received ≥3 prior therapies, and were classified as heavily pretreated. The histological subtypes included 13 patients with follicular lymphoma (FL), 1 patient with small lymphocytic leukemia, and 2 with marginal zone lymphoma. Most (14/16) patients received prior rituximab, 7 patients had rituximab-refractory disease (defined as no response or response of ≤6 months after last rituximab), and 5 patients had received prior radioimmunotherapy. Responses were achieved in 14 patients (75%), 7/10 (70%) heavily pretreated patients, and 4/7 patients (57%) with rituximab-refractory disease. Notably, 85% of patients with FL responded to therapy including 38.4% of patients with a complete response (CR). Median PFS for all patients was 12 months; however, median PFS for patients who received >5 cycles has not been reached. Fatigue (13%), neutropenia (19%), lymphopenia (25%), and hyponatremia (19%) were the most common grade 3 or 4 adverse events. After prophylaxis was initiated, TLS was not observed at the 20 mg dose level. *Conclusions.* These results demonstrate that the R2 combination has significant clinical activity in patients with relapsed/refractory indolent NHL. With an ORR of 85%, patients with FL particularly benefitted from therapy. These promising results have prompted further exploration of R2 combination therapy in larger studies and in earlier lines of therapy.

0296

PLERIXAFOR AND G-CSF FOR PBSC MOBILIZATION IN POOR MOBILIZER PATIENTS WITH LYMPHOMA TREATED WITH MULTIPLE LINES OF THERAPY

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Background. Peripheral blood stem cell (PBSC) mobilization is an important step in the subset of patients (pts) with non-Hodgkin's Lymphoma and Hodgkin's disease who need autologous stem cell transplantation (ASCT). However, a significant percentage of pts are unable to mobilize adequate number of PBSC after conventional stem cell mobilization programs and can not proceed to ASCT. Plerixafor (AMD3100; Mozobil), a bicyclam antagonist of the SDF-1alpha/CXCR4 complex, has been previously reported to augment PBSC collection in pts undergoing PBSC mobilization. *Aim.* To asses the efficacy of plerixafor and G-CSF in achieving the minimum required dose of CD34$^+$ cells for ASCT (2×10^6 CD34$^+$ cells/Kg) in pts with lymphoma who previously failed previous attempts of PBSC mobilization with conventional schemes of chemotherapy + G-CSF. *Methods.* 24 heavily pre-treated or treatment-refractory lymphoma pts classified as "poor mobilizers"were enrolled in a program of compassionate use of plerixafor between 2008 and 2009 in 6 italian centres of REL (Rete Ematologica Lombarda, Lombardy Hematology Network). Pts were defined as poor mobilizers when the concentration of PB CD34$^+$ cells was consistently lower than 10/μL during the recovery phase after chemotherapy and/or when the number of PBSC collected was inadequate for ASCT. *Results.* 10 pts were males and 14 were females. 21 were affected with non-Hodgkin's lymphomas (10 diffuse large B cell lymphoma, 5 follicular lymphoma, 3 mantle cell lymphoma, 1 lymphoplasmocytic lymphoma, 1 peripheral T-cell lymphoma, 1 anaplastic large cell lymphoma) and 3 with Hodgkin's disease. Median age at plerixafor treatment was 50 years (range 27-70). Median number of previous lines of therapy was 3 (range 1-5). Median number of previous attempts of mobilizations with chemotherapy + G-CSF was 2 (range 1-3) (1 attempt in 8, 2 attempts in 12, 3 attempts in 4); 3 pts had received radiotherapy, 4 radioimmunoconjugated antibodies and 3 purine analogues. Median level of CD34$^+$ cells/μL attained with 45 prior mobilization attempts with chemotherapy plus G-CSF was 5/μL. Pts received G-CSF (10 μg/kg/day) for 4 days, followed by daily plerixafor (0.24 mg/kg) plus G-CSF and apheresis for up to 4 days. Overall, plerixafor administration was safe and no serious adverse events were registered. Common plerixafor-related adverse events was ere mild nausea in 10 pts. The median number of circulating CD34$^+$ cells/μL following plerixafor was 8.5×10^6/μL (range 1.7-52 ×10^6/μL). It was more than 10×10^6/μL in 10 pts and more than 20 ×10^6/μL in 7 pts. Ten pts (42%) were able to collect the minimum required dose for ASCT (2×10^6 CD34$^+$ cells/Kg) with a median of 1 apheresis procedure (range 1-4) and 2/10 pts collected more than 4×10^6 CD34$^+$ cells/Kg. *Conclusions.* these results show that plerixafor combined with G-CSF allows collection of adequate number of PBSC in a significant proportion of poor mobilizer, heavily pre-treated pts with non-Hodgkin's lymphoma or Hodgkin's disease, who need consolidation with ASCT.

0297

A MULTICENTER TRIAL ASSESSING FEASIBILITY AND EFFICACY OF A COMBINATION OF HIGH-DOSE METHOTREXATE, CYTARABINE AND THIOTEPA IN PATIENTS WITH PRIMARY CNS LYMPHOMA: IMPACT ON OUTCOME OF CYTARABINE DOSE

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Background. The combination of high-dose methotrexate (HD-MTX) and high-dose cytarabine (HD-araC) (experimental arm of the IELSG #20 trial) has been associated with a significantly better outcome with respect to HD-MTX alone in patients aged ≤75 years with newly diagnosed primary central nervous system lymphoma (PCNSL) [Ferreri AJM, et al. Lancet 2009; 374: 1512-20]. The addition of alkylating agents could improve MTX-araC efficacy since they are active against quiescent G0 cells and they increase cytotoxicity of antimetabolites. Thus, we conducted an exploratory trial addressing a combination (MAT regimen) of HD-MTX, HD-araC and thiotepa, an alkylating agent with excellent CNS bioavailability, in patients with PCNSL. With respect to the experimental arm of the IELSG #20 trial, araC dose was reduced from 2 g/mq to 1 g/mq to minimize the risk of toxicity. *Aims.* To assess tolerability, activity and efficacy of MAT regimen and to compare these results to those observed in the MTX/araC arm of the IELSG #20 trial. *Methods.* HIV-negative patients with newly diagnosed PCNSL, selected by using the same eligibility criteria of the IELSG #20 trial (age 18-75, ECOG-PS≤3, measurable disease), and diagnosed in five Italian and one Swiss centers during 2008 were considered. Patients received 4 courses (interval 3 weeks) of MTX 3.5 g/mq d1 + araC 1 g/mq x 2/d, d2-3 + thiotepa 30 mg/mq d4, followed by whole-brain radiotherapy (WBRT). *Results.* Twenty patients (median age 57 ys; range 42-74) were treated with MAT regimen. No significant differences in patient characteristics between the two groups were observed (Table).

Table.

	MAT (n=20)	MTX+araC (n= 39)	P (Fisher exact)
Median Age (range)	57 ys. (42-74)	59 ys (25-74)	0.98
IELSG risk			
Low	7 (35%)	10 (26%)	0.95
Intermediate	8 (40%)	24 (62%)	
High	5 (25%)	5 (13%)	
Positive CSF cytology	3 (15%)	3/34 (9%)	0.65
Ocular involvement	0 (0%)	4/35 (11%)	0.28
Toxic deaths	1 (5%)	3 (8%)	1.00
Complete remission	4 (20%)	18 (46%)	0.04
Overall response	7 (35%)	27 (69%)	0.01
Stable/Progressive disease	12 (60%)	9 (23%)	-
2-year PFS	20 ± 9%	42 ± 8%	0.03
2-yr OS	22 ± 9%	56 ± 8%	0.01

The comparison between MAT and MTX/araC regimens did not show any significant difference in terms of actually delivered courses (69% vs. 76%), chemotherapy interruptions (65% vs. 44%), dose reductions (60% vs. 44%), G4 neutropenia (85% vs. 74%), G4 thrombocytopenia (85% vs. 64%), infections (45% vs. 30%), G4 non-hematological toxicities (15% vs. 8%), and toxic deaths (5% vs. 8%). Conversely, response rates after MAT chemotherapy (CRR: 20% and ORR: 35%) were significantly lower than those reported with MTX/araC combination (Table). The seven patients who responded to MAT received consolidation WBRT, with early progressive disease (PD) in three cases and systemic dissemination in one; only four of the 12 patients with PD after MAT received WBRT, with no benefit in three of them. At a median follow-up of 20 months, 17 (85%) MAT patients experienced failure (PD, relapse, death), with a 2-yr PFS of 20±9% (Table). These results are significantly worse than those reported with MTX/araC (median f-up: 30 months). Relapse/progression involved the brain in 15 (75%) patients, meninges in one (5%) and abdominal lymphnodes in one (5%). Five patients treated with MAT and 18 treated with MTX/araC are alive, with a 2-yr OS of 22±9% and 56±8%, respectively. *Conclusions.* When compared to MTX/araC standard combination, araC dose reduction did not improve feasibility and was associated with lower efficacy, hiding a potential benefit of the addition of thiotepa in MAT regimen. Four doses of araC 2 g/mq are recommended in the upfront treatment of PCNSL.

0298

SHOULD CEREBROSPINAL FLUID ANALYSIS BE RECOMMENDED ONLY FOR HIGH RISK PATIENT WITH CENTRAL NERVOUS SYSTEM RELAPSE AS AN ESSENTIAL WORKUP IN DIFFUSE LARGE B-CELL LYMPHOMA ?

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Background. In newly diagnosed diffuse large B-cell lymphoma (DLBCL), cerebrospinal fluid (CSF) analysis is currently recommended in patients with risk factors for central nervous system (CNS) relapse, including involvement of paranasal sinus, testis, epidural space, bone marrow or 2 or more extranodal sites. However, there is no satisfactory predicting CNS involvement at initial diagnosis. *Patients and Methods.* We analyzed CSF in 100 patients with newly diagnosed DLBCL from January 2006 to May 2009 at our institution. CSF tests were not directed based on clinical risk factors. *Results.* Among 100 patients (45 female, 55 male), 25 patients (25%) showed leptomeningeal involvement. The involvement was not associated with disease sites (nasal/paranasal sinus, testis, orbit, bone marrow) or the number of extranodal sites. International Prognostic Index score of 3 or greater was not significantly associated with leptomeningeal involvement (P=0.096). We observed no significant difference in the frequency of CNS involvement in patients with or without any of following: CNS-near sites, 2 or more extranodal sites, bone marrow or testis, advanced stage or elevated serum lactate dehydrogenase (18 of 76 (27.7%) vs 7 of 24 (29.2%), P=0.597). *Conclusions.* Our study showed that the leptomeningeal involvement at the initial diagnosis was not significantly associated with risk factors for CNS relapse. Therefore, all the patients with newly diagnosed DLBCL should be considered for CSF analysis as an essential workup in addition to criteria based on the high risks for CNS relapse. Further studies are required to confirme these findings.

0299

PHASE II STUDY OF SMILE CHEMOTHERAPY FOR NEWLY-DIAGNOSED STAGE IV, RELAPSED OR REFRACTORY EXTRANODAL NK/T-CELL LYMPHOMA, NASAL TYPE: NKTSG STUDY

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Background. Extranodal NK/T-cell lymphoma, nasal type (ENKL) is a rare subtype of lymphoma and its standard therapy has not been established. CHOP (-like) chemotherapy has limited efficacy in ENKL, with an overall response rate (ORR) of 36% for newly-diagnosed stage IV diseases and less than 10% for relapsed or refractory diseases. Our previous phase I trial of a new chemotherapeutic regimen, SMILE [Steroid=dexamethasone 40 mg/day d2-4 IV, Methotrexate 2 g/m^2 d1

6hr IV, Ifosfamide 1.5 g/m^2 d2-4 IV, L-asparaginase 6000 U/m^2 d8,10,12,14,16,18,20 IV, and Etoposide 100 mg/m^2 d2-4 IV; every 28 days] showed promising results (Cancer Sci, 2008). *Aims.* To explore a more effective treatment for ENKL, we conducted a phase II study of SMILE chemotherapy. *Methods.* Patients with newly-diagnosed stage IV, relapsed or refractory disease after first-line chemotherapy, between 15-69 years old and with a PS of 0-2 were eligible. Primary endpoint was ORR after 2 cycles of SMILE chemotherapy, and target enrollment was 38 patients. To ameliorate myelotoxicity and based on the results of phase I study, G-CSF was started from day 6. *Results.* From July 2007 to October 2009, 39 patients were enrolled. Pt characteristics were age: 16-67 years (median 47); male:female=21:18; newly-diagnosed stage IV disease in 21; first relapse in 13; and primary refractory disease in 5. Since the first 2 patients died of grade 5 infection, we made a protocol revision stipulating awareness for infection and including lymphocyte count 500/cmm or more into the eligibility criteria. After that, no treatment-related death was observed. Twenty-nine patients (74%) completed the planned treatment. The responses were complete remission (CR) in 15, partial remission in 14, no response in 3, progressive disease in 3, and early death in 4. ORR and%CR were 74% (95% CI, 58-87) and 38%, respectively. Grade 4 neutropenia was common. Grade 4 non-hematologic toxicities were infection (n=5), hyperbilirubinemia (n=1), ALT elevation (n=2), and encephalopathy (n=1). The most common grade 3 non-hematologic toxicity was infection (41%). *Conclusions.* Our results indicate that SMILE chemotherapy is an effective treatment for newly-diagnosed stage IV, relapsed or refractory ENKL. Myelosuppression and infection during the treatment should carefully be managed.

0300

PRALATREXATE EFFICACY AND TOLERABILITY IN PATIENTS WITH RELAPSED OR REFRACTORY CUTANEOUS T-CELL LYMPHOMA (CTCL)

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Background. Pralatrexate is a targeted antifolate designed for preferential uptake and accumulation in tumor cells, due to high affinity for reduced folate carrier and efficient polyglutamation by folypolyglutamyl synthetase. Pralatrexate was recently approved by the Food and Drug Administration for relapsed or refractory peripheral T-cell lymphoma and is currently under evaluation in other tumor types, including CTCL. PDX-010 is a dose-finding single-arm, open-label, multicenter study evaluating pralatrexate in relapsed/refractory CTCL. A dose de-escalating strategy was used to determine if an active and very low toxicity dose/schedule was achievable in this patient population. *Methods.* Eligibility included mycosis fungoides, Sézary syndrome, and primary cutaneous anaplastic large cell lymphoma, with disease progression after at least 1 prior systemic therapy and written informed consent prior to treatment. The starting dose and schedule was 30 mg/m^2 of pralatrexate by IV push weekly for 3 of 4 weeks. If toxicity as defined per protocol was observed, subsequent cohorts received reduced weekly doses (20, 15, 10 mg/m^2) and/or schedules (3/4 or 2/3 weeks). All patients received supplementation with vitamin B12 1 mg intramuscularly every 8-10 weeks and folic acid 1 mg orally QD. Response was evaluated by the modified severity weighted adjustment tool (SWAT) every 2 cycles for 6 months and then every 4 cycles. For patients with lymph node involvement, scans were completed at baseline and upon clinical response or end of treatment, whichever occurred first. *Results.* Thirty-one patients were sequentially enrolled in 6 cohorts and treated at varying doses/schedules. The optimal dose and schedule was identified as 15 mg/m^2 x 3/4 weeks, based on the tolerability and efficacy observed in the initial 6 patients (overall response rate [ORR] of 50%). The response rate (RR) for patients treated at a dose of 15 mg/m^2 x 3/4 weeks or higher was 11/18 (61%). The RR for those treated at lower dose cohorts was 1/13 (8%). The 15 mg/m^2 x 3/4 weeks cohort was expanded and 23 additional patients were treated at the optimal dose (N=29), of which 22 are currently evaluable for response. In the 47 evaluable patients across all cohorts to date, the median number of prior systemic therapies is 4 (range 1-11). Patients received pralatrexate for a median of 3 cycles (range 1-34). The ORR for all cohorts was 40% (19/47), 17 partial responses and 2 complete responses. The responding patients received a median of 4 cycles of treatment. Among evaluable patients receiving the optimal 15-mg/m^2 dose on the 3/4 week schedule, the RR was 45% (10/22). Seven patients in this cohort are on treatment and not yet evaluable. In the expanded cohort, most patients had grade 2 (6/22, 27%) or lower toxicities, with only 2 patients experiencing grade 3 mucositis, and no grade 4 toxicities were observed. Updated efficacy and safety data will be presented. *Conclusions.* Pralatrexate shows impressive activity with minimal toxicity in patients with relapsed/refractory CTCL at the identified optimal dose of 15 mg/m^2 x 3/4 week. Further follow-up is ongoing to assess final efficacy and tolerability.

0301

CLINICOPATHOLOGIC FEATURES OF AGGRESSIVE NK CELL LEUKEMIA (ANKL) - A JAPAN-KOREA MULTI-CENTER STUDY FOR ANKL -

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Backgrounds. Aggressive NK cell leukemia (ANKL) is a malignant disease of mature NK cells that is more common in East Asia and is often associated with EB virus. Because of its rarity, the characteristics of this disease are still largely uncertain. *Aims.* To better elucidate clinicopathologic characteristics of ANKL. *Methods.* A retrospective Japan-Korea multi-center survey of ANKL (ANKL07), was performed. Eligibility criteria are based on the diagnosis for ANKL according to the WHO classification. *Results.* 41 cases were registered from eight institutions. After central reviews, 34 cases were further analyzed, while seven cases were excluded. The main reason of exclusion was a possibility of T cell nature. Median age of the cases was 45 years old (range 16-79) with 26 males and 8 females. Four cases had a history of preceding disorders including EBV-LPD of childhood, mosquito bite hypersensitivity or EBV-associated liver damage. Of note, three female cases presented during pregnancy. Hepatosplenomegaly was recognized in 71% of the cases. Lymphadenopathy and cavity effusions were occasionally seen. Based on the central review, three types on ANKL were morphologically categorized. Type I was defined as LGL closely resembling normal LGL with slightly basophilic cytoplasm. Type III was associated with pleomorphic appearance together with basophilic cytoplasm and bizarre nucleus containing one or more nucleoli. Type II showed the intermediate morphology between type I and III. Cases having mixture of cells with type I and type III were also categorized as type II. Using this classification, there were 11, 13 and 10 cases for type I, II and III, respectively. However, no difference in clinical features except hemophagocytosis was found among the 3 types. Immunophenotypes were similar, typically, CD2$^+$, surfaceCD3$^-$, CD4$^-$, CD5$^-$, CD8$^-$, CD16+, CD56$^+$, and CD57$^-$. EBV was positive in 85% of the patients. Median survival was 51 days (range; 1-1,630). No difference of the survival was recognized among the type I, II and III. Median ratio of tumor cells in peripheral blood (PB) and bone marrow (BM) was 8% and 22%, respectively. 11 cases showed leukemic cells less than 20% both in PB and BM. The clinical characteristics and prognosis of these cases did not differ from patients with more tumor cells in PB/BM. This suggests that main infiltration sites of tumor cells are liver, spleen and BM irrespective of leukemic nature. *Summary*. These results clarify ANKL as a mature NK cell proliferative disease with heterogenous morphologies associated with EBV. ANKL shows markedly poor prognosis, regardless the PB/BM tumor burden.

0302

A NATION-WIDE SURVEY OF HTLV-1-ASSOCIATED ADULT T-CELL LEUKEMIA/LYMPHOMA IN JAPAN

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Background. Adult T-cell leukemia/lymphoma (ATLL) is caused by the first discovered human oncogenic retrovirus, human T-cell leukemia virus type-1 (HTLV-1). The prevalence of HTLV-1 varies in different area of the world, and HTLV-1 carriers are clustered in the southwest-

ern districts of Japan, as well as among native Andeans, North Iranians, Central Africans, and African descendants in the Caribbean and South America. Clinical and laboratory features of ATLL are similar in different countries, whereas there is a significant difference in age of patients between countries. *Aims.* To show current incidence, mortality, and clinical features of patients with ATLL in Japan, and to compare the results with the results obtain from previous studies performed nationwide in Japan and report from other countries. *Methods.* A nation-wide survey of ATLL and B-cell non-Hodgkin lymphoma (B-NHL) as an internal control was performed in 2009. Questionnaires for registration were distributed to physicians in 479 hospitals having the department of hematology.*Results.* A total of 910 cases of ATLL and 7,164 cases of B-NHL, newly diagnosed from January 2006 to December 2007 (2 years), were registered from 156 hospitals. Male-female ratios were 1.16 for ATLL and 1.22 for B-NHL. Among all ATLL cases registered, 59.8% were from Kyushu, a HTLV-1 endemic area of Japan, and the ratio of ATLL to B-NHL in this area was 1 to 4, while that in Tokyo was 1 to 50. According to the classification criteria for the subtypes of ATLL, 46.7% was classified as acute type, 34.8% as lymphoma type, 10.3% as smoldering type, and 8.2% as chronic type, which results were different from those obtained from previous studies, and the ratio of acute type was decreased with an increase of lymphoma type. The more significant difference was found in age of patients. The mean age gradually increased from 58.3 years in the 5th nation-wide survey (1988-1989) to 60.3 years in the 8th nation-wide survey (1994-1995) and finally to 65.2 years in the present study (range from 19 to 94, median 67). The mean age of Japanese patients is now more than 20 years older than that reported from Jamaica and Brazil (43 years). Patients younger than 55 years, an upper age limit for allogeneic hematopoietic stem cell transplantation, are now only 19.2% of the total patients. Increase of the mean age is explained by a high HTLV-1 prevalence (>10%) in elderly people over 60 years in Japan and by a continuing development of ATLL from this large pool of HTLV-1 carriers. According to the mortality statistics from the Ministry of Health, Labor and Welfare Japan, approximately 1,000 people died annually from ATLL, which did not change in the past decade. The estimated annual incidence of ATLL calculated from the number of B-NHL as an internal control was 1,150 cases.SUMMARY: A nation-wide survey showed a change of ATLL subtypes and an increase of the age of patients, which require a change of therapeutic strategy. The incidence of ATLL in Japan was estimated to be 1,150 cases per year.

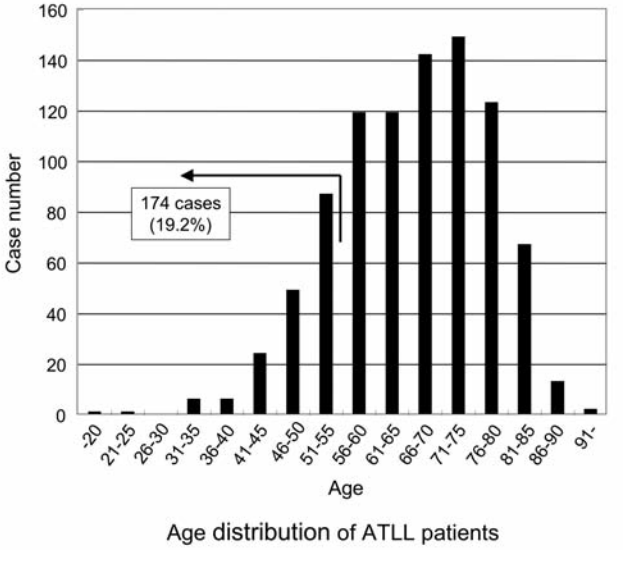

Figure.

0303

DIFFERENT PROGNOSTIC SCORES IN PERIPHERAL T-CELL LYMPHOMA (PTCL)

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Background. PTCLs comprise a heterogeneous group of lymphoid neoplasms with poor prognosis. In contrast to aggressive B-cell lymphomas, PTCLs have not a specific clinical, immunophenotypic, genetic or gene expression signature of prognostic significance. A number of prognostic scores have been used in PTCLs, The International Prognostic Index [IPI], the Prognostic International Index [PIT], the PIT model modified by Went *et al.* [mPIT] and The International PTCL Project score [IPTCLP]. All of them have been shown to predict the outcome of the patients with PTCL. However, a systematic comparative study of all these models in a homogeneous series of patients have never been addressed before. *Aims.* to study the main clinico-biological features and outcome of patients with PTCLs from a single institution and to compare the predicitive value of different prognostic scores (IPI, PIT, mPIT IPTCLP). *Methods.* One hundred twenty one non-immunocompromised patients (73M/47F; median age, 55 years) diagnosed with PTCL according to the WHO classification, in a single institution between March 1990 and October 2008. The distribution according to the histologies was: PTCL "not other-wise specified"(PTCL-NOS) 56 (47%), anaplastic large cell lymphoma ALK$^+$ (ALCL) 21 (17%), angioimmunoblastic T-cell lymphoma (AITL) 19 (16%), NKTCL 15 (12%), hepatosplenic T-cell lymphoma (HSTL) 7 (6%) and subcutaneous panniculitis-like T-cell lymphoma (SPTCL) 3 (2%). Patients with ALK$^+$ALCL were analyzed separately because of its particular behavior. The median follow-up was 3.9 years (range, 2.8 to 5) for surviving patients, and the overall characteristics for the patients included performances status (PS) >1, 40%; stage III-IV, 81%; bone marrow (BM) infiltration 39%; any extranodal involvement, 76%; high serum LDH, 56%; platelet count <150×10^9/L, 28% and 56 (72%) patients of 78 with available data had high β2-microglobulin levels. Ninety one (91%) patients received adriamycin-containing chemotherapy, including 21 (21%) patients whom received high dose of chemotherapy and a stem cell transplantation as part of the first line therapy. The variables used to calculate the different scores were the previously established: IPI [age, performance status (PS), LDH, Ann Arbor stage and extranodal involvement], PIT [age, PS, LDH and bone marrow involvement], IPTCLP [age, PS and platelet count] and mPIT [age, PS, LDH and Ki-67 index]. The main clinico-biological and evolutive variables were analyzed. *Results.* The distribution of the patients according to the risk group, complete response rate and overall survival (OS) are detailed in Table 1.

Table 1. Complete response and overall survival according in the different scores.

Prognostic scores	*N (%)*	*CR rate (%)*	*5 year OS (%)*
Whole group	100	36	25
IPI			
Low risk	25 (25)	36	45
Low intermediate risk	23 (23)	33	28
High intermediate risk	30 (30)	17	21
High risk	22 (22)	14	0
PIT			
Low risk	16 (16)	25	75
Low intermediate risk	26 (26)	31	26
High intermediate risk	35 (35)	33	17
High risk	23 (23)	11	0
IPTCLP			
Low risk	29 (29)	31	58
Low intermediate risk	44 (44)	52	15
High intermediate risk	20 (20)	14	5
High risk	7 (7)	3	0
mPIT*			
Low risk	24 (58)	57	38
Intermediate risk	8 (20)	7	0
High risk	9 (22)	36	0

Regarding response, IPI (low vs. intermediate/high) was the best score to predict CR achievement (RR=2.45; P=0.05). IPTCLP (low vs. intermediate/high) was the best model to predict OS in a a Cox mutivariate analysis comparing the four models [IPI, PIT, IPTCLP and mPIT]

(RR=3.52 ; P<0.0001). These results were similar when only the subset of PTCL-NOS was selected. *Conclusions.* All the scores demonstrated its useful to predict the outcome of patients with PTCL, but IPTCLP demonstrated to be the most significance score to predict OS.

0304

RETROSPECTIVE ANALYSIS FOR RISK FACTORS FOR PATIENTS WITH PERIPHERAL T-CELL LYMPHOMA IN HOKKAIDO, JAPAN

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Background. Peripheral T-cell lymphomas (PTCLs) are heterogeneous disease with a dismal prognosis. To evaluate the incidence, treatment and prognosis of PTCLs, Hokkaido Clinical Hematology Forum conducted a multicenter retrospective survey in Hokkaido, Japan. *Patients and Methods.* Clinical data for patients with PTCLs were collected from 13 hospitals in Hokkaido, Japan, and data for patients who met the following criteria were analyzed: diagnosed between 2002 and 2008, aged more than 15 years and diagnosed as having mature T-cell neoplasms defined by the new WHO classification 3rd edition. Totally, data for 272 patients were collected and the diagnoses were adult T-cell leukemia/lymphoma (ATL, 33%), PTCL-unspecified (PTCL-u, 25%), NK-cell neoplasms (13%), angioimmunoblastic T-cell lymphoma (AITL, 13%), anaplastic large cell lymphoma (ALCL, 7%) and other subtypes (10%). After excluding patients with ATL and NK-cell tumors due to the different strategy of treatment, data for 142 patients with other subtypes of PTCLs were analyzed for characteristics and prognosis. *Results.* The median age of the patients was 67.5 years. Many patients had the following risk factors: stage III-IV (88%), high LDH (63%), B symptoms (41%) and IPI high/high-Int risk (56%). Eighty-nine percent of the patients received chemotherapy as a primary treatment and most of the patients received a CHOP-like regimen; however, 37% of them received a reduced dose due to advanced age and approximately half of the patients could not complete the treatment plan, mainly due to disease progression and complications. Overall response rate was 67.4% and CR rate was only 41.4%. In multivariate analysis, bone marrow involvement (odds ratio: 4.7) and the number of treatment cycle (<5, odds ratio: 11.1) were determined as risk factors for induction failure. In patients who achieved CR, 45.1% of the patients relapsed. Patients with ALCL who achieved CR did not relapse and patients with IPI low/low-int risk were at low risk for relapse (33% vs. high/high-int risk, 65%, P=0.03). Hematopoietic stem cell transplantation (SCT) was performed in 22 patients [autologous (auto) SCT: n=14, reduced-intensity allogeneic (allo) SCT: n=8]. Median age was younger in the allo group (49 years vs. 57 years) and other characteristics were also different in the auto group and allo group: patients with PTCL-u usually received auto-SCT, while patients with CTCL received allo-SCT, allo group had higher risk of IPI and greater chemresistance. Only one patient died after SCT. Survival of patients who received SCT was better than that of patients who did not receive SCT (P<0.01). After median follow-up of 2.8 years, 2-year overall survival rate (OS) and 2-year progression-free survival rate were 55% and 32%, respectively. Ninety-six percent of the patients died with lymphoma. B symptoms at diagnosis (HR 2.2), induction failure after primary treatment (HR: 2.9) and SCT not being performed (HR: 4.7) were revealed to be risk factors for OS by multivariate analysis. *Conclusions.* The prognosis of PTCLs is poor. Therefore, more effective regimen as a primary treatment than a CHOP-like regimen is needed, and SCT should be considered as an upfront setting for patients who are at high risk for relapse.

0305

PRALATREXATE ACTIVITY IN PATIENTS WITH RELAPSED/REFRACTORY PERIPHERAL T-CELL LYMPHOMA (PTCL): RELATIONSHIP BETWEEN RESPONSE AT CYCLE 1 AND SUBSEQUENT SURVIVAL

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Background. Pralatrexate is a targeted antifolate designed for preferential uptake and accumulation in tumor cells, due to high affinity for the reduced folate carrier-1 protein and efficient polyglutamation by folypolyglutamyl synthetase. Pralatrexate was approved by the US Food and Drug Administration for the treatment of patients with relapsed/refractory PTCL based on the results of the pivotal PROPEL trial. The primary end point, objective response rate (ORR) using International Workshop Criteria, was evaluated by both independent central review (ICR) as well as by the investigator. The aim of the present analysis was to determine if responses observed early in the course of treatment are prognostic for survival. *Methods.* The relationship between tumor response and survival was tested using a landmark analysis, where patients alive and evaluated for response at the landmark time point were categorized as either having responded or not responded. The landmark time point was study day 53, which coincides approximately with the end of cycle 1. Survival was subsequently measured to the landmark and compared between categories via the Cox model. *Results.* The ORR (best response) was 29% (32/109) per ICR and 39% (43/109) per investigator assessment. By the landmark time point, there were 90 patients with an assessment per ICR, and 95 with an assessment per investigator. Of these, there were 20 responders per ICR and 33 responders per investigator. Responders and nonresponders were matched on demographics (gender, race, age, weight) and disease characteristics (mean time since diagnosis, histopathology per central review, baseline cutaneous involvement, ECOG performance status, number of prior regimens, number of prior systemic regimens). Overall survival was 14.5 months. Results of the landmark analysis are presented in Table 1. *Conclusions.* Patients treated with pralatrexate who experienced tumor response during cycle 1 had a 31% (per central review) and 54% (per investigator) reduction in risk of death. As assessed by the investigators, early responses to pralatrexate are strongly correlated with prolonged survival (P=.01) as seen from the landmark analysis.

Table 1.

	Per ICR (N = 90)		Per Investigators (N = 95)	
	Responders	Nonresponders	Responders	Nonresponders
N	20	70	33	62
Median Subsequent Survival	17.6 mos	13.4 mos	21.3 mos	8.6 mos
Hazard Ratio (95% CI)*	0.69 (0.33, 1.43)		0.46 (0.25, 0.84)	
P	.32		.01	

*Hazard ratio for risk of death subsequent to landmark responder:nonresponder.

0306

NOVEL MECHANISM OF TRANSPLACENTAL CANCER TRANSMISSION: NATURAL-KILLER/T CELL LYMPHOMA IN THE PARATESTICULAR REGION OF AN INFANT OF MATERNAL ORIGIN

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Introduction. The placental syncytiotrophoblast layer is a barrier that protects the fetus from infection and cancer metastasis. Even if mother-derived cells enter the fetus, they are usually rejected and cannot be engrafted unless the infant is immunocompromised. Materno-fetal transmission of leukemia/lymphoma is therefore extremely rare. Based on the following case findings, we propose a novel mechanism for the vertical transmission of cancer.Case report A male infant born at 30 weeks gestation weighing 1659 g maintained good health until 7 months of age. At age 8 months, his scrotum appeared enlarged and was referred to Nihon University Hospital. MRI examination revealed a testicular tumor and the infant underwent left orchiectomy. Immunohistochemical analysis of paraffin-embedded sections showed that tumor cells expressed LCA, TdT, CD3, and CD56, but not CD10, CD20, CD79a, CD34, CD68, or ALK. *in situ* hybridization analysis further revealed that the tumor cells were positive for Epstein-Barr-virus-encoded small RNA (EBER). The diagnosis was EBV-associated natural-killer (NK)/T cell lymphoma, which is a very rare histology in children. Multi-agent chemotherapy was initiated and after completing 4 courses he received cord blood transplantation. The infant's mother was a 32-year-old Japanese woman whose pregnancy was unremarkable until the 7th month of gestation. Two weeks before delivery, she was transported to Nihon University Hospital because of petechia and fever. Laboratory findings showed thrombocytopenia (68000/μL) and liver dysfunction (GOT 513 U/mL, T. Bil 6.72 mg/d, LDH 5681 U/mL). HELLP syndrome was diagnosed and emergency cesarean section was performed; however, her condition deteriorated after delivery. A bone marrow smear showed hypoplasia with large granular lymphocytes expressing cytoplasmic CD3 and CD56. Although she was treated with high-dose steroids, etoposide and cyclosporine, hepatic failure and disseminated intravascular coagulation developed and she died on postpartum day 25. Since a high EBV genome load (2.8×10^6 copies/μg DNA of peripheral $CD8^+$ lymphocytes) was detected, she was diagnosed with EBV-related NK/T cell leukemia, which is prevalent in East Asia and often fatal. Due to the similarities in disease phenotype between the mother and the infant, identification of the origin of the infant's lymphoma was attempted using two independent *Methods.* fluorescence *in situ* hybridization and microsatellite analysis. Procedures were carried out according to the Declaration of Helsinki and with the informed consent of the family. As a result, the infant's tumor cells were found to be identical to the leukemic cells of the mother.Discussion Recently, it was demonstrated that maternal leukemic cells are not rejected by an infant when they have lost non-inherited maternal HLA antigens [Isoda T, et al. PNAS; 106: 17882-5]. In our case, maternal leukemic cells most likely transmigrated into the paratesticular area where they engrafted and proliferated because the testis is a sanctuary site protected from the immuno-surveillance system. We propose that this is a novel mechanism for the vertical metastasis of cancer.

0307

LONG-TERM OUTCOME OF PATIENTS WITH PERIPHERAL T-CELL LYMPHOMA TREATED WITH FIRST-LINE INTENSIVE CHEMOTHERAPY WITH AUTOLOGOUS STEM CELL TRANSPLANTATION

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Background. Peripheral T-cell lymphomas (PTL) are infrequent subtypes of non-Hodgkin's lymphomas. The clinical course is aggressive, and despite anthracycline-based multiagent chemotherapy, the median survival is about 2-3 years. An optimal first-line chemotherapy protocol is not established and the role of high-dose therapy with autologous stem cell transplantation (SCT) is still unclear. *Aim.* To analyze the long-term outcome of unselected PTL patients treated with intensive first-line sequential chemotherapy with high-dose therapy and autologous transplant consolidation. *Method*. The sequential chemotherapy (SQ) protocol consists of 3 cycles of CHOEP-21-like regimen (PACEBO), 1 cycle of an ifosfamide and methotrexate-based regimen (IVAM) and a priming regimen with high-dose cytosine arabinoside (HAM). Consolidation is provided with myeloablative conditioning (BEAM 200) and autologous SCT. Here we report our experience with 29 patients with PTL. The histological subtypes were as follows: peripheral T-cell lymphoma, not otherwise specified (PTCL, NOS) n=13; anaplastic large cell lymphoma (ALCL) ALK-negative n=5; ALCL ALK-positive n=3; ALCL with an unknown ALK status n=3; angioimmunoblastic lymphoma n=1; hepatosplenic lymphoma n=1; Sézary syndrome n=1; and enteropathy-associated T-cell lymphoma (EATL) n=2. The median age at diagnosis was 48 years (29-64), most patients had advanced Ann Arbor stages (22 patients, 77%), IPI score ≥3 was found in 13 (45%) and PIT score ≥2 in 17 (59%) of the 29 patients. Twenty-six patients underwent first-line therapy according to the SQ protocol, two patients received the ProMACE-CytaBOM regimen and one patient was treated according to the modified SQ protocol. Eighteen patients received first-line high-dose therapy and autologous SCT consolidation; two patients were consolidated with allogeneic SCT in the 1st complete remission and one patient in the 1st relapse. Treatment responses were assessed according to the International Workshop Criteria (Cheson, 1999). Twelve patients with FDG-avid lymphoma were examined with integrated PET/CT at the time of diagnosis and after first-line therapy. *Results.* Nineteen (66%) patients achieved CR, 3 (10%) partial remission and 7 (24%) patients failed the procedure. The overall response rate was 76%. PET negativity (complete metabolic response) after therapy was achieved in 8/12 (75%) individuals. After a median follow-up of 55.1 months, 14 (48.3%) patients relapsed or progressed (8 PTCL NOS; 2 ALCL ALK-positive; 2 ALCL ALK-negative; 1 Sézary syndrome; 1 EATL; median time 16.1 months) and nine patients died (lymphoma progression). Eleven patients (50% of chemosensitive patients) survived more than 50 months. Three of the long-term survivors were treated with allogeneic SCT. The 2-year progression-free survival (PFS) was 52% (95%CI, 0.33-0.71); the 2-year overall survival (OS) rate reached 65% (95%CI, 0.47-0.84). PET negativity was associated with a lower probability of relapse (chi-square P=0.09). *Conclusions.* Our data show that intensive first-line therapy with etoposide-based regimens and autologous transplant consolidation may lead to long-term disease control in about a half of patients with chemosensitive PTL. Patients with partial treatment response may be treated with allogeneic stem cell transplantation. Achieving of PET negativity is probably an essential prerequisite for long-term CR.

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Myelodysplastic syndromes 1

0308

DIAGNOSTIC USE OF MULTIPARAMETER FLOW CYTOMETRY IN PATIENTS WITH SUSPECTED MYELODYSPLASTIC SYNDROME: EVALUATION OF 1013 CASES AND CORRELATION TO CYTOMORPHOLOGY, CYTOGENETIC, AND CLINICAL DATA

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Background. Diagnosis and classification of myelodysplastic syndromes (MDS) is based on cytomorpholgy (CM) and cytogenetics (CG). Multiparameter flow cytometry (MFC) may add important information. *Aims.* To evaluate the diagnostic role of MFC in MDS. *Methods.* We analyzed 1013 cases with suspected MDS by CM, CG, and MFC in parallel. *Results.* Cases were classified by CM as RA (n=31), RARS (n=27), RCMD (n=64), RCMD-RS (n=49), RAEB-1 (n=133), RAEB-2 (n=81), 5q- syndrome (n=24), CMML (n=65), MDS unspecified (MDS-u, n=15), MDS borderline to AML (MDS/AML, n=6), MDS/myeloproliferative neplasia overlap (MDS/MPN, n=16), suspected MDS (n=225), reactive condition (n=266), and normal findings (n=11). CG findings were normal karyotype (n=768), isolated deletion of long arm of chromosome 5 (del(5q), n=43), isolated aberrations of chromosome 7 (n=14), isolated trisomy 8 (n=30), isolated deletion of long arm of chromosome 20 (del(20q), n=21), complex aberrations (n=23), loss of Y-chromosome (n=43), other aberrations (n=71). Concordance between CM and MFC was 82.0% for diagnostic results in 788 cases with unequivocal CM. 277 of these 788 cases were classified by CM as no MDS, 13 (4.7%) of which showed MDS-typical features by MFC. Additional 225 cases showed only minor dysplastic features by CM, 51 (22.7%) of which showed clear evidence of MDS by MFC. We then focused on cytogenetically aberrant cases without unequivocal MDS by CM. In 6/12 (50.0%) cases with no MDS by CM and MDS-typical cytogenetic aberrations MFC revealed MDS characteristics. In another 11/23 (47.8%) cases with minor dysplastic features by CM and MDS-typical cytogenetic aberrations MFC revealed MDS characteristics. Furthermore, we compared blasts counts as determined by CM and MFC and found a strong correlation (P<0.001) although the mean±SD percentage was higher as determined by CM as compared to MFC (4.67±4.18 vs. 3.78±2.97). Frequencies of aberrantly expressed antigens significantly differed between cases rated by CM as MDS (median number of aberrantly expressed antigens: 3), suspected MDS (1), and no MDS (0, P<0.001). In various cases MFC identified MDS-typical aberrant antigen expression in cell compartments not rated dysplastic by CM. Particularly, aberrant antigen expression did not strongly correlate to dysgranulopoiesis by CM. Thus, in 406 cases without dysgranulopoiesis by CM dysplastic features were observed with the following frequencies: aberrant CD13/CD16 expression pattern: 104 (25.6%); aberrant CD11b/CD16 expression pattern: 62 (15.3%); CD56 expression: 38 (9.4%); CD33 negativity: 44 (10.8%); CD64 negativity: 2 (0.5%). An aberrant expression of ≥2 antigens in granulocytes was found in 16/31 (51.6%) and 15/27 (55.6%) of cases with RA and RARS, respectively. Ovedrall, spearman rank correlation confirmed a highly significant relation between the number of aberrantly expressed antigens and IPSS (r=0.409, P<0.001). In 257 cases with data on overall survival (OS) the presence of MDS-related findings (≥3 aberrantly expressed antigens, a blast count >5% in MFC, or a reduced side-scatter signal) resulted in significantly inferior 6-year-OS (68% vs. 100% P=0.008). *Conclusions.* The present analysis clearly demonstrates a diagnostic yield of MFC in addition to CM and CG in cases with suspected MDS.

0309

AN EVALUATION OF THE ASSOCIATION OF CDKN2B (P15) DNA METHYLATION WITH RESPONSE AND SURVIVAL IN PATIENTS WITH HIGHER-RISK MYELODYSPLASTIC SYNDROMES (MDS) TREATED IN THE AZA-001 TRIAL

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Background. The phase III AZA-001 study in patients with higher-risk MDS demonstrated that treatment with azacitidine resulted in significantly improved overall survival (OS) compared with conventional care regimens (CCR) (Fenaux, Lancet Oncol, 2009;10:223). A previous study in a small number of similar risk patients showed that CDKN2B methylation status was predictive of response to azacitidine, with lower baseline methylation observed in responders vs non-responders (Raj, Leukemia, 2007;21:1937). *Aims.* We therefore sought to determine whether CDKN2B promoter DNA methylation density in bone marrow samples at baseline from patients on AZA-001 treated with azacitidine or CCR was associated with OS and response. *Methods.* The percentage of DNA methylation was determined for 18 CpG units in the CDKN2B promoter of genomic DNA isolated from 295 pre-treatment bone marrow aspirates of consenting patients using the Sequenom EpiTYPER® platform. A weighted average of methylation levels in these 18 CpG units was used to evaluate association with clinical outcome, with the number of individual CpG dinucleotides comprising each CpG unit used as the weight for that unit. Kaplan-Meier methods were used for survival estimates. Cox proportional hazards models, stratified by the randomization factors FAB subtype and international prognostic scoring system (IPSS) group, were used to estimate hazard ratios (HRs) and associated 95% confidence intervals (CIs). Adjustments were made for the following covariates: ECOG performance status, LDH, hemoglobin, number of previous red-blood-cell transfusions, and presence or absence of the cytogenetic -7/del(7q) abnormality. *Results.* (Table): In 295 patients (azacitidine [n=148] and CCR [n=147]) the median weighted average methylation density of 18 CpG units in the CDKN2B promoter DNA was 13.6% (IQR: 10.4-23.5%), ranging from 3.1 to 64.6%, and was similar between treatment arms (16.0% [IQR: 10.6-24.8%] for azacitidine and 13.1% [IQR: 10.1-21.3%] for CCR). When groups were dichotomized at the median of 13.6% methylation, there was an OS benefit for both azacitidine methylation groups (≤13.6% and >13.6%) compared with the respective methylation groups of patients treated with CCR. Relative to the low methylation group (≤13.6%) treated with CCR, the HRs for risk of death were 0.63 [95% CI: 0.39-1.01] for the azacitidine ≤13.6% group, 0.55 [95% CI 0.35-0.84] for the azacitidine >13.6% group, and 0.95 [95% CI 0.62-1.47] for the CCR >13.6% group. Additionally, there was a higher proportion of patients with hematologic response (CR+PR+HI [IWG 2000]) for both methylation groups (≤13.6% and >13.6%) treated with azacitidine compared with the respective methylation groups treated with CCR. *Summary/Conclusions.* These data show that the OS and hematologic response benefits observed with azacitidine vs CCR were independent of baseline methylation status of CDKN2B, and that a similar proportion of patients experienced increased hematologic response rates with azacitidine in each methylation group.

Table 1.

CDKN2B Methylation Weighted Average	Overall Survival			
	Deaths (n/N)	K-M Median (months)	Hazard Ratio (95%CI)	p-value
CCR, ≤ 13.6%	47/80	15.21	1.00	
CCR, > 13.6%	43/67	17.02	0.95 (0.62 – 1.47)	0.830
AZA, ≤ 13.6%	28/68	Not reached	0.63 (0.39 – 1.01)	0.056
AZA, > 13.6%	41/80	18.39	0.55 (0.35 – 0.84)	0.007
	Hematologic Response			
	CR+PR+HI* n (%)	CR+PR† n (%)	HI† n (%)	
CCR, ≤ 13.6%	34 (42.5)	12 (15.0)	28 (35.0)	
CCR, > 13.6%	21 (31.3)	6 (9.0)	17 (25.4)	
AZA, ≤ 13.6%	37 (54.4)	19 (27.9)	34 (50.0)	
AZA, > 13.6%	37 (46.3)	24 (30.0)	36 (45.0)	

*Patients were counted only once according to best response. †Patients may have been included in both categories (CR+PR and HI), if they had achieved both responses.

0310

THE IMPACT OF THE DEGREE OF ANEMIA ON SURVIVAL OF PATIENTS WITH MYELODYSPLASTIC SYNDROME. A BASIS FOR PROGNOSTIC ASSESSMENT AND CLINICAL DECISION MAKING

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Background. More than 90 percent of patients with myelodysplastic syndrome (MDS) are anemic at the time of diagnosis, while moderate

to severe anemia is observed in approximately 50 percent of cases. In MDS, low hemoglobin levels have been found to result in cardiac remodeling, and the onset of a regular transfusion requirement is associated with a higher risk of heart failure and significant worsening of survival. *Aims.* In this study we aimed at evaluating the prognostic impact of the degree of anemia in MDS patients classified according to WHO criteria, as a basis for objective prognostic assessment and clinical decision-making. *Methods.* To this purpose, we longitudinally monitored the decrease in hemoglobin (Hb) in 920 patients with MDS, followed at the Department of Hematology, S. Matteo Hospital, University of Pavia, Italy. Hb level was grouped in categories of 1 g/dL (from >13 g/dL to <8 g/dL for males and from >12 g/dL to <8 g/dL for females). Sex-specific uni- and multivariable analyses with time-dependent covariates were performed by means of Cox proportional hazards regression models. *Results.* As a first step, we performed a Cox univariate time-dependent survival analysis stratified by sex. A decrease in Hb was associated with a progressive worsening of overall survival (OS): statistical significance was obtained beginning from Hb <11 g/dL in males (HR from 5.2 to 15.7, P values from .029 to <.001) and from Hb <9 g/dL in females (HR from 6.3 to 11.8, P values from .013 to <.001). We then performed sex-specific multivariable Cox regression analyses including age, Hb categories, WHO subgroups, cytogenetic risk groups categorized according to IPSS, number of cytopenias, cardiac comorbidity as time-dependent covariates, and found a significantly higher risk for Hb <9 g/dL in males (HR 5.6, P=.018) and <8 g/dL in females (HR=5.6 P=.026). These Hb thresholds were also found to be associated with a significantly higher risk of cardiac death (HR 8.1, P=.004). Finally, we aimed at assessing whether these sex-specific Hb thresholds could be as effective as transfusion-dependency in the prognostic assessment of MDS patients. The unfavorable prognostic impact of the sex-specific Hb thresholds was comparable to that of transfusion-dependency in both univariable (HR=4.0, P<.001, and HR 4.1, P<.001, respectively) and multivariable time-dependent Cox regression (HR=2.6, P<.001, and HR=2.5, P<.001, respectively). The substantial equivalence of these two models was confirmed by the Akaike information criterion (AIC, 2967 versus 2970, respectively). Based on these results, we recalculated WPSS risk groups, using sex-specific Hb thresholds instead of red cell transfusion requirement, and obtained highly concordant risk categories (Kendall tau coefficient 0.94). Moreover, results by both Cox regression and Kaplan-Meier time-dependent analyses were superimposable. *Conclusions.* The findings of this study confirm the independent unfavorable effect of severity of anemia on survival of MDS patients. In the calculation of WPSS, the use of sex-specific Hb thresholds instead of transfusion-requirement provides a more objective score computation, and - compared to the original model - allows to identify highly concordant risk groups with similar survival and risk of leukemic progression.

0311

EFFECT OF BASELINE EPO AND PRIOR ERYTHROPOIESIS STIMULATING AGENTS ON RBC TRANSFUSION INDEPENDENCE IN LOW-/INT-1-RISK MDS WITH DEL5Q TREATED WITH LENALIDOMIDE: A RANDOMIZED PHASE 3 STUDY (MDS-004)

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Background. MDS patients with erythropoietin (EPO) levels >500 mIU/mL and transfusion burden ≥2 units/month have poor response to erythropoiesis stimulating agents (ESAs). The randomized, phase 3, multicenter, double-blind MDS-004 study in RBC transfusion-dependent patients with Low- or Int-1-risk MDS with del5q demonstrated that lenalidomide (LEN) 10 mg and 5 mg achieved significant RBC transfusion independence (TI) versus placebo. RBC-TI (56% versus 41%) and cytogenetic response (41% versus 17%) rates were higher with LEN 10 mg. *Aim.* This sub-analysis aims to investigate RBC-TI according to baseline EPO levels and prior ESA use in LEN-treated patients, and the effect of the LEN dose used in those patient subgroups. *Methods.* Patients who provided informed consent received LEN 10 mg (days 1-21 of a 28-day cycle) or LEN 5 mg (days 1-28 of a 28-day cycle); concomitant ESAs were prohibited. RBC-TI for ≥26 consecutive weeks was assessed according to baseline EPO levels (≤500 mIU/mL and >500 mIU/mL) and prior ESA use. *Results.* 87 patients (LEN 10 mg: n=41; LEN 5 mg: n=46) were included. Median RBC transfusion burden was 6 units/month (range 1-28). In the LEN groups combined, RBC-TI rates were similar in patients with EPO ≤500 mIU/mL (48%) versus >500 mIU/mL (51%; P=0.81) (Table 1). In patients with EPO >500 mIU/mL, the RBC-TI rate was higher with LEN 10 mg versus LEN 5 mg (76% versus 29%; P=0.0016), whereas the RBC-TI rate was similar between LEN doses in patients with EPO ≤500 mIU/mL (43% versus 54%; P=0.57). In the LEN groups combined, RBC-TI rates were lower with prior ESA (36%) versus no prior ESA (63%; P=0.01). RBC-TI rates were similar in the LEN 10 mg versus 5 mg groups in patients with prior ESA (42% versus 30%; P=0.42) and without prior ESA (76% versus 52%; P=0.12). In the LEN groups combined, RBC-TI rates in patients who had an EPO level >500 mIU/mL or received prior ESA were lower than those with EPO ≤500 mIU/mL and no prior ESA use (42% versus 80%; P=0.025). RBC-TI rates were similar with LEN 10 mg and 5 mg in patients with EPO >500 mIU/mL or who had received prior ESA (51% versus 33%; P=0.12) and in those with EPO ≤500 mIU/mL and no prior ESA (80% versus 80%; P=1.0). No differences in time to RBC-TI and duration of RBC-TI occurred according to baseline EPO level and prior ESA use, irrespective of LEN dose. *Conclusions.* LEN is effective in achieving RBC-TI regardless of baseline EPO level or prior ESA use; LEN 10 mg achieved higher RBC-TI rates than LEN 5 mg in patients with baseline EPO level >500 mIU/mL, and to a lesser extent in patients with prior ESA treatment who represent refractory populations.

Table 1.

	LEN 5 mg (n=46)	LEN 10 mg (n=41)
EPO, n (%)		
≤500	13 (28)	14 (34)
>500	24 (52)	21 (51)
Prior ESA, n (%)		
Yes	23 (50)	24 (59)
No	23 (50)	17 (41)
EPO ≤500 and no prior ESA, n (%)	5 (11)	5 (12)
EPO >500 or prior ESA, n (%)	36 (78)	35 (85)
RBC-TI, % (95% CI)		
EPO ≤500	54 (25.1–80.8)	43 (17.7–71.1)
EPO >500	29 (12.6–51.1)	76 (52.8–91.8)
Prior ESA	30 (13.2–52.9)	42 (22.1–63.4)
No prior ESA	52 (31.7–72.6)	76 (56.3–96.6)
EPO ≤500 and no prior ESA	80 (28.3–99.5)	80 (28.3–99.5)
EPO >500 or prior ESA	33 (18.6–51.0)	51 (34.0–68.6)

Baseline EPO levels are reported as mIU/ML. CI, confidence interval.

0312

MOLECULAR PATHOGENESIS OF REFRACTORY ANEMIA WITH RINGED SIDEROBLASTS

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Background. Refractory anaemia with ringed sideroblasts (RARS) is characterized by anaemia, erythroid apoptosis, cytochrome c release and mitochondrial ferritin accumulation. To dissect the molecular mechanisms underlying the RARS phenotype, we have determined the gene expression profiles of erythroblasts generated from normal (NBM) and RARS marrow CD34$^+$ cells. Pathway analysis showed that genes involved in apoptosis, erythroid survival and mitochondrial function were altered. *Aim.* To test the hypothesis that RARS is caused by alterations in a gene involved in cellular iron transport or possibly mitochondrial function. *Results.* We confirmed the microarray data by quantitative real-time PCR (qRT-PCR). Several erythropoiesis-associated genes were deregulated in RARS CD34$^+$ cells. ABCB7, transporting iron from mitochondria to cytosol and associated with inherited ring sideroblast formation was severely downregulated compared to normal bone marrow. Moreover, ABCB7 expression decreases dramatically in RARS progenitors undergoing erythroid differentiation, but increases with differentiation in normal bone marrow cultures. The same pattern was observed for the mitochondrial integrity gene MFN2. To study the function of candidate genes, K562 cells were employed which can be induced to erythroid differentiation by hemin treatment. The expression of ABCB7 in K562 cells by lentiviral transduction facilitated the

expression of γ-globin and glycophorin A, indicating that ABCB7 potentiated erythroid differentiation. Further, BM CD34+ cells from three RARS patient were transduced with lentiviral vectors expressing ABCB7-YFP. The transduction efficiency was tested by flow cytometry. Colony forming cell assay was initiated from day 3 cultures. The number of erythroid colonies, myeloid colonies, YFP+ erythroid, and YFP+ myeloid colonies were counted on day 14 after transduction. The number of colonies generated from mock vector-transduced cells was in comparison with that from untransduced cells, the percentage of YFP+ colonies was in consistence with that tested by flow cytometry. While colony number increased significantly from the abcb7 vector-transduced cell cultures. Interestingly, YFP+ colonies from the ABCB7 vector-transduced cell cultures increased considerably. Results indicated that upregulation of ABCB7 increased the clonogenic capacity of RARS progenitors. In addition, qRT-PCR results from day 10 cell culture samples of one RARS patient showed that upregulation of ABCB7 reduced the accumulation of mitochondrial ferritin. *Summary*. Using pathway analysis with clinical and cell biological knowledge, we can limit the number of candidate genes from the vast amount of data obtained from global gene expression analysis. With lentiviral transduction we can increase the number of genes tested on somatic hemotopoietic stem cells. In the absence of ABCB7 mutations or hypermethylation, we hypothesize that upstream mechanisms with impact on cellular iron transport may be involved in RARS pathogenesis.

0313

DETECTION OF TP53 MUTATIONS IN LOW-RISK MYELODYSPLASTIC SYNDROMES WITH DEL(5Q) IS ASSOCIATED WITH DISEASE PROGRESSION

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Background. TP53 mutations have been identified as a strong prognostic marker in myelodysplastic syndromes (MDS). However, mutations have been identified preferentially in high risk MDS and often occur in conjunction with complex chromosomal abnormalities. We recently described a patient with 5q- syndrome that at time of transformation carried a del(17p) and a TP53 mutation. Intriguingly, we were able to demonstrate the mutation already two years prior to transformation. This represents a novel finding in 5q- syndrome and suggests an unrecognized heterogeneity. Therefore we hypothesize that TP53 mutated subclones at an early stage of MDS with del(5q) may be associated with an increased risk of disease progression. *Aims.* To investigate the frequency of TP53 mutations in low risk MDS with a del(5q) and to assess the association with disease progression. *Patients and Methods*. Thirty-eight sequential patients with low-risk MDS and del(5q) from the Karolinska Hospital were included (33 isolated del[5q] and 5 del[5q]+1). Nineteen patients (50%) were transfusion-dependent at time of diagnosis. Twenty-three patients (61%) received treatment with lenalidomide. All sequential bone marrow samples from diagnosis and during follow-up were reviewed and stained for p53 protein. Mutational analysis of TP53 was performed using deep sequencing in 34 patients. The Kaplan-Meier estimate and the logrank test were used for analysis of survival and disease progression.

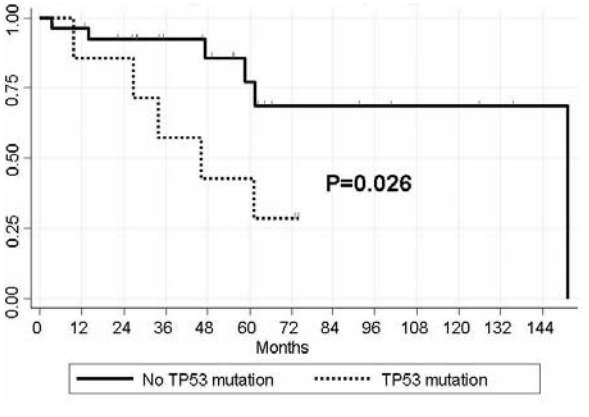

Figure 1. Probability of disease progression.

Results. The median follow-up was 48 months (interquartile range 24-66). Pre-progression TP53 mutations were found in 7 of 34 patients (21%), at levels of 3-55%. Overexpression of p53 protein was associated with the presence of TP53 mutation; 5 of 7 patients with mutated subclones showed at least 2% strongly p53 positive cells in the marrow, while none with less than 2% p53 positive cells were mutated. The presence of p53 mutation was significantly associated with disease progression (defined as a blast count above 10% or acquisition of a complex karyotype; P=0.026; Figure 1). There was no significant difference in overall survival (P=0.95). Due to the low number of mutated patients it was not possible determine any potential influence of treatment with lenalidomide on outcome. *Conclusion.* Mutations of the p53 gene at an early stage of low-risk MDS with del(5q) was associated with disease progression. If this can be confirmed in a larger cohort of patients it may constitute an important prognostic marker that could influence clinical decision making.

0314

INDEPENDENT IMPACT OF TRANSFUSION DEPENDENCY AND IRON OVERLOAD ON SURVIVAL OF PATIENTS WITH MYELODISPLASTIC SYNDROMES

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Background. Patients with myelodysplastic syndromes (MDS), especially those with a lower-risk type of disease, are prone to developing iron overload as a result of chronic blood transfusion therapy, and also partly due to increased duodenal iron absorption induced by ineffective erythropoiesis. Transfusion dependency is clearly associated with a decreased probability of survival (Malcovati L *et al.* J Clin Oncol 2007; 25:3505). Additional recent data suggest that iron overload could also influence outcome independently fashion from transfusion dependency. *Aim.* The main aim of this study was to validate the independent prognostic value of iron overload and transfusion dependency in a relatively large single-centre series of MDS patients. *Methods.* We retrospectively analyzed the outcome of 639 patients diagnosed with *de novo* MDS according to FAB criteria between 1994 and June 2009 at the Catalan Institute of Oncology in Barcelona. Iron overload was defined as serum ferritin level >1.000 ng/mL, and transfusion dependency was defined by the WHO-based Prognostic Scoring System (WPSS). Actuarial curves of overall survival used Kaplan-Meier method and compared by log-rank test. Multivariate analysis of overall survival were performed by Cox proportional hazards regression method. *Results.* Median age at diagnosis was 71 years (range: 25-99); 410 male and 229 female. MDS subtype distribution was as follows: RA 113 (18%), RARS 91 (14%), RCMD 99 (16%), RAEB-1 126 (20%), RAEB-2 43 (7%), LAM (RAEB-t) 13 (2%), CMMoL 135 (21%), MDS-U and MDS with isolated 5q- 14 (2%).

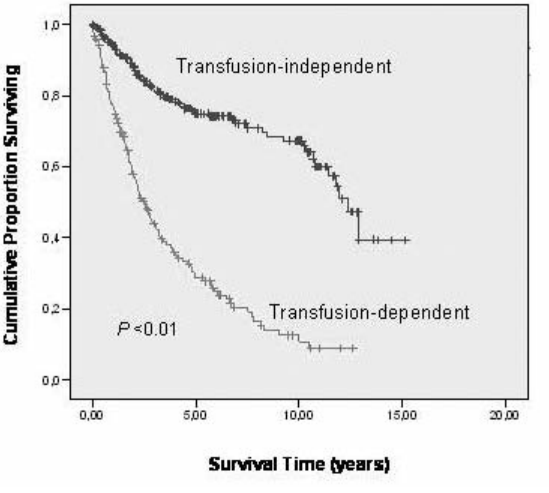

Figure 1.

The median follow-up time for these patients was 49 months (range: 8 to 182). Cytogenetic analysis was available at diagnosis for 199 (31%)

patients, in whom IPSS could be assessed (39% low risk, 42% intermediate 1, 13% intermediate 2, 6% high risk). Complete transfusion history was available for all patients. Two hundred and seventy patients (42%) were transfusion-dependent, requiring a median of 2.03 (range: 0.5-13.2) packed red blood cell monthly transfusions. Iron overload, as determined by serum ferritin level, was available in 195 patients (31%). Univariate analysis of factors influencing overall survival identified transfusion dependency, iron overload, age, karyotype risk, percentage of blasts at diagnosis, number of cytopenias, IPSS scoring system, WPSS scoring system, hemoglobin <10 g/dL and platelets <100×10^9/L as statistically significant factors (all P<0.001). Median overall survival was for transfusion-dependent patients compared with transfusion independent ones was 34 vs 56 months (P<0.001) and median survival of patients with high ferritin levels was significantly shorter than that of patients with ferritin <1.000 (34 vs 53 years; P<0.001). Multivariate analysis confirmed in our single-centre series that transfusion dependency (RR=5.8; P<0.001) and iron overload (RR=2.1; P<0.025) were strongly associated with overall survival and added independent prognostic information. *Conclusions.* Our results confirm an independent prognostic value of iron overload and transfusion dependency on overall survival in patients with MDS, and further suggest that strategies to reduce iron overload may have a positive effect on overall survival in these patients.

0315

ALLOGENEIC STEM CELL TRANSPLANTATION FOR MDS WITH BONE MARROW FIBROSIS

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Background. Bone Marrow Fibrosis has an important impact on the prognosis of patients with MDS. *Aims and Methods.* We evaluate the impact of bone marrow fibrosis in 721 patients who underwent allogeneic hematopoietic stem cell transplantation (HSCT) for MDS/AML and were reported to the EBMT. *Results.* No fibrosis was noted in 483 pts, mild or moderate fibrosis in 199 pts and severe fibrosis in 39 pts. Diagnosis in the none, mild/moderate and severe fibrosis group were RA/RARS (36%, 39% and 30%), RAEB (43%, 43% and 48%) and RAEB-t (21%, 18% and 20%). Stem cell source were from related (63%, 63% and 62%) or unrelated (36%, 36% and 38%) donors. Leukocyte engraftment (>1.0×10^6/L) was observed after a median of 16, 17 and 20 days for the none, mild/moderate and severe fibrosis group, respectively (P=0.002). Incidence of acute or chronic GvHD did not differ between the groups. Non-relapse mortality at 1 year was comparable in all groups (26% vs 30% vs31%, P=0.3). Cumulative incidence of relapse at 3 years was higher in the severe fibrosis group in comparison to mild/moderate and none fibrosis group (47% vs 27% vs 28%, P=0.04), resulting in significant worse 3 year overall survival in the severe fibrosis group (21% vs 40% vs 49%, P=0.002). In a multivariate analysis severe bone marrow fibrosis remained an independent risk factor for survival (HR 1.9, P=006). Other factors for reduced survival were advanced disease at transplant (HR 1.97 P=0.005), non-CR before transplant (HR 1.88, P<0.001) and HLA-mismatch (HR 1.48, P= 0.02). I *Summary.* The negative impact of mild or moderate bone marrow fibrosis in MDS patients can be overcome by allogeneic stem cell transplantation, but severe bone marrow fibrosis remained an independent risk factor for reduced survival after transplantation.

0316

IMPACT OF LENALIDOMIDE ON HEALTH-RELATED QUALITY OF LIFE IN PATIENTS WITH RBC TRANSFUSION-DEPENDENT LOW- OR INT-1-RISK MYELODYSPLASTIC SYNDROMES WITH DEL5Q: A RANDOMIZED PHASE 3 STUDY (MDS-004)

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Background. In a randomized, phase 3, multicenter, double-blind study (MDS-004), lenalidomide (LEN; 5 mg and 10 mg) was generally well tolerated and resulted in significant RBC transfusion independence (TI) and cytogenetic response in patients with Low- or Int-1-risk myelodysplastic syndromes with del5q. Significant improvements in hemoglobin levels (median maximum hemoglobin increase: LEN 10 mg = 6.3 g/dL; LEN 5 mg = 5.1 g/dL) were also observed. *Aim.* To present health-related quality of life (HRQoL) analyses from the MDS-004 study. *Methods.* Patients who provided informed consent were randomized to LEN 5 mg on days 1-28 or LEN 10 mg on days 1-21, both of a 28-day cycle, or placebo. Patients with at least minor erythroid response at 16 weeks continued double-blind treatment for up to 52 weeks, until erythroid relapse or disease progression. Placebo and LEN 5 mg patients who did not respond by week 16 were considered treatment failures and could crossover to LEN 5 mg or 10 mg, respectively, in an open-label extension phase. Patient-reported HRQoL was assessed using the Functional Assessment of Cancer Therapy-Anemia (FACT-An) questionnaire. FACT-An comprises FACT-general plus additional items that assess the impact of fatigue and other anemia-related symptoms. The minimal clinically important difference in FACT-An scores is 7 points (Cella et al., J Pain Symptom Manage. 2002). FACT-An was administered at baseline, and weeks 12, 24, 36, and 48. ANOVA compared the change from baseline in FACT-An scores at week 12 in each LEN group versus placebo. Longitudinal assessment of FACT-An scores through week 48 for patients randomized to lenalidomide treatment is presented. Double-blind data are reported. *Results.* Overall, 205 patients (median age 68 y, range 36-86 y) were randomized (LEN 10 mg: n=69; LEN 5 mg: n=69; placebo: n=67) and included in the intent-to-treat analysis. FACT-An scores were available at both baseline and week 12 for 71% of randomized patients (LEN 10 mg: n=48; LEN 5 mg: n=46; placebo: n=51). Among patients with FACT-An scores at both baseline and week 12, the mean (SD) baseline FACT-An scores were 121.0 (20.1), 125.3 (23.4), and 121.3 (26.6) for the LEN 10 mg, LEN 5 mg, and placebo groups, respectively. There was a significant improvement in HRQoL with LEN 10 mg versus placebo at week 12 (mean change scores 5.2 versus -'3.3, respectively; P=0.030). The difference between LEN 5 mg versus placebo followed a similar trend (mean change scores 3.4 versus -'3.3, respectively; P=0.09). Absolute change from baseline FACT-An scores in LEN-treated patients exceeded 7 points at weeks 24, 36, and 48 (Figure). Of patients with available FACT-An scores, > 75% overall (> 90% in the LEN 10 mg group beyond week 16) achieved RBC-TI. *Conclusions.* Improvements in HRQoL, as measured by the FACT-An relative to placebo, were apparent at week 12 and were greatest in the LEN 10 mg group. Clinically relevant HRQoL improvements relative to baseline were reported in both LEN groups beyond week 12.

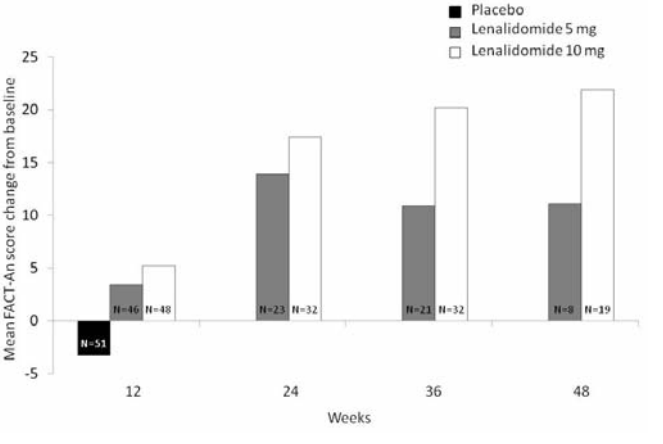

Figure.

0317

GENE EXPRESSION ANALYSIS IN DIFFERENT HEMATOPOIETIC CELL COMPARTMENTS IDENTIFIES COMMONLY DOWN REGULATED GENES AND SUGGESTS A POSSIBLE ROLE OF AN IMPAIRED HELICASE ACTIVITY IN MDS

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Background. Several genetic and molecular genetic alterations have been described in myelodysplastic syndromes (MDS) but the exact underlying mechanisms remain elusive. Furthermore, gene expression analysis identified critical genes that could be involved in initiation and progression of MDS. However, whether the underlying defects affect the myeloid clone only or whether bone marrow stroma cells and

peripheral blood mononuclear cells derive from the malignant clone as well remains controversial. *Aims.* In order to further elucidate whether an underlying defect might translate into an altered gene expression pattern in different cell compartments we performed gene expression analysis on purified CD34+ bone marrow cells, bone marrow stromal cells (BMSC) and mononuclear peripheral blood cells (PB) from patients with MDS and healthy controls. *Methods.* Heparinized bone marrow samples were obtained after informed consent from 18 MDS patients (5 RA, 2 RARS, 10 RAEB and 1 RAEB-t) and 7 healthy controls. CD34+ hematopoietic progenitor cells were isolated by high gradient magnetic cell separation (Miltenyi Biotech, Bergisch Gladbach, Germany). BMSC were obtained by expansion of adherent bone marrow cells in specific cell culture. RNA was extracted from CD34+ cells, BMSC and PB using TRIzol reagent (Invitrogen, Life Technologies, Grand Island, NY) according to the manufacturer's protocol. Quality controlled RNA was hybridized according to the standard Affymetrix protocol to HG-U133 Plus 2.0 microarrays. Data analysis was performed using the Gene Spring Software version 4.0 (Silicon genetics, San Carlos, CA). Restrictions were set as follows: only genes that were 'present' in 100% of samples were used for further analyses, genes were considered as 'differentially expressed' when they showed at least 2-fold change between the different groups. Statistical significance was calculated by non-parametric t-test, with P<0.05. Gene ontology analysis was performed by using the public DAVID database and only genes that showed an enrichment score of at least 1 were considered. *Results.* Comparison of gene expression patterns in CD34+ cells, BMSC and PB from MDS patients to healthy volunteers identified 133 probe sets that were at least 2-fold downregulated in MDS. Interestingly, none were comonly upregulated in MDS in all three cell compartments. The 133 probe sets comprised of 124 genes that were further analyzed for gene ontology by using the public DAVID database. Biological functions that were most frequently affected included RNA transport as well as transcription regulation. Among these genes we identified the SMARCC1 gene exclusively being downregulated in MDS in all three cell compartments. SMARCC1 encodes for a protein which is a member of the SWI/SNF family displaying helicase and ATPase activities. These proteins are thought to regulate transcription of certain genes by altering the chromatin structure. *Summary*. In conclusion, gene expression analysis identified a possible key gene which is affected in three different cell compartments in MDS and that has been shown to play a critical role in sustaining genomic stability in human cells. Thus, impaired expression of SMARCC1 might result in an increased genomic instability, a feature which is thought to play a pivotal role in the initiation and progression of MDS.

0318

IDH1 MUTATIONS IN PATIENTS WITH MYELODYSPLASTIC SYNDROMES ARE ASSOCIATED WITH AN UNFAVORABLE PROGNOSIS

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Background. Myelodysplastic syndromes (MDS) are a heterogeneous group of hematopoietic stem cell disorders with a high propensity to transform to acute myeloid leukemia (AML). The molecular changes that can lead to MDS as well as the progression to AML are still poorly understood. Heterozygous missense mutations in IDH1 at position R132 have recently been described in AML. However, the incidence of IDH1 mutations has not been reported in MDS. *Aim.* The aim of this study was to investigate the incidence and prognostic impact of IDH1 and IDH2 mutations in MDS. *Methods.* In the present study, we examined 246 patients with MDS or AML with myelodysplasia-related changes for mutations in IDH1 (R132), IDH2 (R172), and NPM1 by direct sequencing. *Results.* We found that mutations in IDH1 occur with a frequency of 3.6 and 7.5 percent in MDS (n=193) and AML with myelodysplasia-related changes (n=53), respectively. All mutations were heterozygous missense mutations, leading in the majority of MDS patients to Arg132Cys substitution. A mutation of IDH2 (R172) was identified in one AML patient, but not in MDS patients. IDH1/2 mutations were not found concurrently with NPM1 mutations in MDS patients. The presence of IDH1 mutations was associated with a shorter overall survival (HR 3.20; 95%CI 1.47-6.99). Patients with mutated IDH1 showed a higher rate of transformation into AML (67% vs 28%, P=0.04). In multivariate analysis including IDH1 mutation status, IPSS score, transfusion dependence, and karyotype, the presence of IDH1 mutation remained an independent unfavorable prognostic marker in MDS (HR 3.57; 95%CI 1.59-8.02; P=0.002). In patients with AML and a history of MDS, the mutational status of IDH1/2 had no prognostic impact on OS (HR 1.82; 95%CI 0.64-5.17; P=0.26). *Conclusions.* We have identified IDH1 mutations of amino acid 132 in 3.6 percent of MDS patients, and found a correlation of mutated IDH1 with unfavorable outcome in these patients.

0319

FEASIBILITY OF HYPOMETHYLATING AGENTS FOLLOWED BY ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANTATION IN PATIENTS WITH MYELODYSPLASTIC SYNDROME

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Background. The role of hypomethylating agent therapy (HMT) as a bridging to allogeneic hematopoietic cell transplantation (HCT) in patients with myelodysplastic syndrome (MDS) still remains to be determined. *Aim.* We investigated the feasibility and clinical efficacy of HMT followed by HCT in patients with MDS at a single institute. *Methods.* Patients with MDS were treated with HMT - azacitidine (AZA; 75mg/m^2 once daily s.c. on day 1 to 7) or decitabine (DEC; 20mg/m2 once daily i.v. on day 1 to 5) every four weeks. Hematologic improvement (HI) was evaluated per each cycle and bone marrow/cytogenetic response were regularly monitored per 2-4 cycles. HCT was performed for patients in their good performance status and with available donor, irrespective of the response to HMT. *Results.* Among 75 patients who were treated with HMT between Sep. 2006 and May 2009, 19 patients of median age 47 years (range, 23-69 years) who received HCT were analyzed. Seven patients (RA: 2, RCMD: 5) were classified as low-risk and 12 patients (RAEB-1: 7, RAEB-2: 5) as high-risk, based on WHO classification at the time of HMT administration. Patients were classified by IPSS score as 'LOW' (n=2), 'INT-1' (n=8), 'INT-2 (n=6) and 'HIGH' (n=2). In terms of HMT, 9 patients were treated with DEC and 10 patients with AZA. There were no significant difference between two groups except in the median number of cycles - 3 (range, 1-6) for DEC and 5.5 (2-13) for AZA. Best response to HMT was 'CR' (11%), 'mCR' (32%), 'SD' (21%), and 'progression' (5%) for 13 evaluable patients. Six patients achieved HI (HI-P for 2, and HI-P/N for 4 patients). After the completion of HMT, there were no changes in the WHO classification compared with pre-HMT status in 15 patients (79%), one patient improved, and 3 patients (16%) progressed to acute myeloid leukemia (AML). 'Consideration of HCT' (48%) was the most common cause of HMT termination, followed by 'no effect' (26%), 'failure' (16%), and 'prolonged cytopenia' (11%). HCT was performed after the median of 7.3 months (range, 3.5-89.7 months) from the diagnosis of MDS, and 6.5 months (range, 2.1-23.9 months) from the initiation of HMT. Most of patients (95%) received non-myeloablative regimen based on fludarabine/busulfan/antithymocyte globulin as a conditioning, peripheral blood-mobilized cell (median cell number of 6.9×10^6) as stem cell source. Neutrophil/platelet engraftment were achieved in 95%/79% of patients, respectively and the number of patients who required transfusion after day 100 was 2 for RBC and 1 for platelet. Cumulative incidence rate of acute/chronic GVHD was 42%/26%, respectively. Post-HCT 2-year relapse-free survival (RFS) rate was 78% and 2-year overall survival (OS) rate was 68%. None of the clinical features associated with MDS / HMT had any significant effect on the outcome except that progression to AML during HMT did on the OS (HR 0.10, 95% CI 0.02-0.59, P=0.012). *Conclusions.* HMT followed by HCT was a feasible and effective treatment strategy for patients with MDS. Further investigations are warranted to prove the improvement of outcomes with the addition of HMT before HCT.

0320

VASCULAR ENDOTHELIAL GROWTH FACTOR OVEREXPRESSION IN BONE MARROW CELLS FROM PATIENTS WITH MYELODYSPLASTIC SYNDROME: BIOLOGICAL AND CLINICAL RELEVANCE

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The expression of various angiogenesis mediators has been found to be altered in myelodysplastic syndrome (MDS) bone marrow and

abnormal angiogenesis has been implicated in the pathogenesis of the disorder. Vascular endothelial growth factor (VEGF) is one of the most important agents to stimulate angiogenesis. Intense coexpression of VEGF and receptor was detected in bone marrow immature myeloid elements from MDS patients and it was hypothesized that this angiogenic glycopeptide may have autocrine and paracrine regulatory effects on the hematopoietic system and contribute to disease progression. We analyzed by immunocytochemistry VEGF expression in bone marrow cells from 211 patients with MDS stratified according to IPSS criteria (134 low risk and 77 high risk patients), not previously treated, and 96 non hemopathic subjects. We also measured by an immunoassay VEGF bone marrow plasma levels as well as the release of VEGF in the supernatants of cell cultures from representative MDS and control cases. Our aims were to evaluate whether abnormalities in the expression of this factor were associated with relevant laboratory or clinical findings and to define their possible prognostic value; moreover, to investigate a possible correlation between VEGF expression levels and various biological parameters such as circulating endothelial cell (CEC) levels, bone marrow microvessel density, apoptosis, proliferation. VEGF was detected in most maturing myeloid cells from control samples (median 25%, IQR 14-44%). In MDS VEGF myeloid levels (median 42%, IQR 30-56%) were higher than those in controls (P<0.0001), and also many erythroblasts expressed VEGF (median 40%, IQR 13-74%). A few MDS $CD34^+$ stem cells (2-16%) expressed VEGF, whereas normal $CD34^+$ cells did not express this factor. The release of VEGF was demonstrated in all samples; VEGF levels were tendentially higher in the media conditioned by MDS mononuclear cells (median 29 pg/mL, IQR 11-77), especially from low-risk patients (median 56 pg/L, IQR 2-88), than in controls (median 9 pg/mL, IQR 0-23), and significantly higher in MDS bone marrow plasma than in normal marrow plasma (P=0.01). No significant relationship was detected between VEGF expression and CEC levels or marrow microvessel density, whereas there was a positive correlation between marrow microvessel density and CECs (P<0.001). In MDS a positive correlation between VEGF myeloid or erythroid levels and apoptotic rate (P=0.02 and P=0.04 respectively) was observed, whereas VEGF expression was independent of the proliferative rate. VEGF cell levels were unrelated to clinical or laboratory features such as age, leukocyte count, blast cell percentage or karyotype. In multivariate analysis including age, WHO subgroups and IPSS variables, myeloid VEGF levels above median values were independently associated with longer overall survival (P=0.03) and evolution-free survival (P=0.04). In conclusion, we have systematically evaluated the relation between VEGF abnormal expression profile and other biological and clinical features in MDS. Rather than stimulate angiogenesis, the production and release of this factor may influence hematopoietic cell death and contribute to ineffective hematopoiesis, possibly by a paracrine induction of inflammatory pro-apoptotic cytokines from endothelial cells and macrophages, with a potential prognostic role.

0321

TRANSFUSION INTENSITY, NOT THE CUMULATIVE NUMBER OF RED BLOOD CELL (RBC) TRANSFUSIONS, IS ASSOCIATED WITH POOR PROGNOSIS IN PATIENTS WITH MYELODYSPLASTIC SYNDROME (MDS) OR CHRONIC MYELOMONOCYTIC LEUKEMIA (CMML)

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Background. Previous studies have revealed an association between the cumulative number of RBC transfusions and shorter survival in patients with MDS, though the causal connection between both features has not yet been fully elucidated. *Aim.* To test three hypothetical reasons for such association: 1) the cumulative number of RBC transfusions is a confounder merely reflecting the time elapsed from diagnosis; 2) it is a surrogate for higher transfusion intensity, which in turn reflects a more severe bone marrow failure, or, alternatively, 3) it is the total amount of transfused RBC units by itself that influences on prognosis. *Methods.* Review of the transfusion history and the clinico-hematological data of 204 transfusion-dependent patients with MDS or CMML seen at our institution from 1995 to 2009. Standard statistical methods were used for survival analysis. *Results.* Median age at first transfusion was 75 (range: 16-99) years and 102 (50%) patients were females. WHO subgroups were refractory cytopenia with multilineage dysplasia (RCMD, n=96), refractory anemia with excess of blasts (RAEB, n=57), CMML (n=25), simple refractory anemia (RA, n=16), RA with ringed sideroblasts (RAS, n=7), and del5q (n=3). Karyotype was available in 175 patients (high risk: 27). At the study closing date, median follow-up from first transfusion was 1.9 years, 138 (68%) patients had died, 8 (4%) were lost to follow-up, 49 evolved into acute leukaemia (AL), and the median number of RBC units transfused per patient was 40 (range: 4-330). Out of the 204 patients, 134 received ≥25 RBC units, at which time the median follow-up from first transfusion was 10 months. Based on the WHO subgroup and cytogenetics, two prognostic groups were defined: good (RA, RAS, del5q, RCMD, with good or intermediate risk cytogenetics; median survival 3.7 years) or bad (RAEB, CMML, or high risk cytogenetics; median survival: 0.7 years). Transfusion intensity was calculated at the time of each transfusion as the number of yearly equivalent RBC units, had the patient kept being transfused at that rhythm for an entire year, and was categorized by quintiles. In order to test the study hypotheses, several nested Cox models were built to predict AL-free survival. In the basal model, the above defined poor prognosis group (hazard ratio [HR]: 2.6, 95%CI: 1.7-4.0, P<0.001) and having received ≥25 RBC units, evaluated as a time-dependent covariate (HR: 3.4, 95%CI: 2.2-5.2, P<0.001), predicted a shorter AL-free survival. Including follow-up ≥10 months from first transfusion as a time-dependent covariate had a remarkable prognostic effect (HR: 12, 95%CI: 3-50, P=0.001) but did not change the prognostic significance of having received ≥25 RBC units. In the next step, adding transfusion intensity to the basal Cox model had a significant effect on AL-free survival (HR: 1.7, 95%CI: 1.4-2.0, P<0.001) and cancelled the prognostic value of having received ≥25 RBC units (HR: 1.3, 95%CI: 0.7-2.2, P=0.33). *Conclusions.* Transfusion intensity, instead of the cumulative amount of RBC units, is the transfusion-related variable really influencing on the prognosis of patients with transfusion-dependent MDS or CMML. Transfusion seems, therefore, to be a surrogate for disease severity.

0322

MESENCHYMAL STEM CELLS FROM LOW-GRADE MDS AND HEALTHY DONORS DISPLAYED DISTINCTIVE GENE EXPRESSION PROFILE PATTERNS, WITH 5Q- SYNDROME AS AN INTERMEDIATE SUBGROUP

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Background. Currently, there is intensive research on the role of mesenchymal stem cells (MSC) in the pathophysiology of inefficient hematopoiesis. We and other groups have shown that MSC from myelodysplastic syndrome (MDS) display morphological, immunophenotypical and genomical abnormalities. However, no transcriptional analysis of MSC from MDS has been described yet. *Aim.* To compare the gene expression profile by microarray approach of MSC from 5q- syndrome, low-grade MDS and healthy controls and to establish a distinctive molecular signature between those groups. *Methods.* Fifteen samples from 5q- syndrome (n=6), other low-grade MDS ([oMDS],n=6; 3 RA, 2 RARS and 1 RCMD) and healthy donors (HD,n=3) were isolated and *in vitro* expanded following standard procedures. After the third passage, total RNA was isolated using TRIzol reagent (Invitrogen) and their integrity was confirmed by the RNA 6000 Nano kit (Agilent Technologies Inc). Labeled samples were hybridized to Affymetrix Human Gene 1.0 ST arrays (Affymetrix Inc). Using SPOTFIRE 9.1 (TIBCO Software Inc), we selected those markers with the following conditions: an ANOVA test between HD, oMDS and 5q- subgroups <0.05 and a fold change value between HD vs. oMDS+5q- >1.5, calculating the whole signature of HD/oMDS/5q- through a hierarchical clustering analysis (HCA). These selected genes were analyzed by the Ingenuity Pathway software (Ingenuity Systems®). Additionally, relative expression of several markers were confirmed by quantitative PCR using commercial TaqMan® assay (Applied Biosystems®). *Results.* Based on 190 selected probesets, we observed three main MSC subgroups in the HCA: one including all HD and three 5q- samples, a second one including the another three 5q- and one RA samples and the third one including the other MSC from low-grade MDS. Similar pattern was obtained when HCA was performed by including all probesets with an ANOVA value >1.5, independently of the fold changes between subgroups. When these 190 probes, including 153 known genes, were analyzed with the Ingenuity software, we observed several affected networks related to cellular movement, cell cycle, DNA replication, recombination, cellular assembly and organization. The main altered functions in MSC from MDS+5q- in comparison to HD were: cellular growth and proliferation, connective tissue development and function and changes in tissue morphology (quantity and density). Genes related to these processes were: ANGPT1, APC, CCND1, ENC1, IL6ST, LIFR, PIK3CA, RB1CC1, RMI1, TRPM7 (overexpressed in MSC from MDS) and BCL6,

COL14A1, DBC1, KLF4, PTGER4, PTGS2, SFRP4, SPRY2, TRIB1,WISP2 (underexpressed in MSC from MDS). Similar results were obtained when cDNA from the same MSC samples were tested with real-time PCR approach. MSC from 5q- patients showed an expression profile slightly different than low-grade MDS (i.e. higher CCND1, and lower ANGPT1, LIFR, PIK3CA, RB1CC1, SLPI and SFRP4) and resembing HD expression profile with similar ANGPT1, IL6ST, LIFR, PIK3CA, RB1CC1, RMI1, SPRY2 expression than normal controls. *Summary*. We conclude that MSC from low-grade MDS showed a distinctive gene expression profile in comparison to those from healthy donors, mainly involving signalling pathways related to proliferation, apoptosis, angiogenesis and multilineage differentiation. Additionally, MSC from 5q- syndrome displayed an intermediate profile between low-grade MDS and healthy controls.

0323

C-CBL MUTATIONS IN DE NOVO MDS AND CMML AT BOTH DIAGNOSIS AND AML TRANSFORMATION: A COMPARATIVE ANALYSIS ON 76 MATCHED PAIRED MARROW SAMPLES

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Background. Somatic C-CBL mutations have been described in chronic myelomonocytic leukemia (CMML), myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) at initial diagnosis. The molecular basis of the progression of MDS or CMML to AML remained to be determined. Recent studies on C-CBL mutations in MDS or CMML were carried out either at initial presentation or only at AML transformation. The role of C-CBL mutations in MDS /CMML to AML transformation is not defined yet. *Aims.* We aimed to analyze C-CBL mutations on paired samples of MDS and CMML at both diagnosis and AML transformation to determine their roles in AML progression and also to correlate the C-CBL mutation status with clinical outcome. *Materials and Methods.* Seventy-six matched paired bone marrow samples including 47 high risk MDS (15 RAEB1, 18 RAEB2 and 14 RCMD)/AML and 29 CMML (17 CMML1 and 12 CMML2)/AML were analyzed for C-CBL mutations. Mutational analysis was performed by DHPLC and/or direct sequencing of all RT-PCR or DNA-PCR products amplified with different primer pairs covering the coding sequences of exon 7 to exon 9 of C-CBL gene. *Results.* Three (one each with RAEB1, RAEB2 and RCMD) of 47 patients with MDS had C-CBL mutations at initial diagnosis, including one homozygous mutation located in Linker region (Y371S) and another two heterozygous mutations located in the Ring finger domain (P418S and G415S). At AML transformation, all 3 patients carrying C-CBL mutations at initial presentation retained the identical C-CBL mutations and one with heterozygous G415S mutation at diagnosis exhibited homozygous pattern at AML phase. Additional 3 patients acquired heterozygous mutations (L399V, L370_Y371 insL, and C416W, respectively) during AML progression. Of the 29 patients with paired CMML/AML samples, 3 had C-CBL mutations at diagnosis, two were homozygous for I383L and I383R. The one with I383L also harbored a heterozygous Q367P mutation at diagnosis, she had a retention of the homozygous I383L but lost Q367P at AML phase. The remaining one had heterozygous G418S at both phases of disease. None of the CMML patients acquired new mutations during AML evolution. Analysis of clinicohematologic variables revealed no significant differences in age, gender, hemoglobin level, platelet count, percentage of blasts in bone marrow or blood, WHO subtype, cytogenetics or IPSS, except that C-CBL mutation-positive patients had a higher WBC count in CMML (P=0.039), but not in MDS. The mutation status did not influence outcome in terms of risk of AML transformation and overall survival in patients with MDS or CMML. *Conclusions.* Our results showed that acquisition of C-CBL mutations might occur during AML evolution in MDS, whereas C-CBL mutations were always present in both CMML and AML clones for individual patients. Clonal expansion was also observed in a subset of AML patients derived from MDS or CMML.

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0324

INTERIM ANALYSIS OF THE USE OF 5-AZACYTIDINE FOR TREATMENT OF MYELODYSPLASTIC SYNDROME/ACUTE MYELOID LEUKAEMIA WITH CHROMOSOME 7 OR COMPLEX CYTOGENETIC ABNORMALITIES

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Background. Chromosome 7 anomalies are well known to confer an adverse prognosis in patients with myelodysplastic syndrome (MDS). Preliminary data from various groups suggested that the use of 5-azacytidine, which is a DNA methyl transferase inhibitor, may improve clinical outcome in this group of poor risk patients. *Aims.* We report on the interim results of an open-label single-centre study designed to evaluate the clinical and haematological outcomes in MDS patients with chromosome 7 anomalies alone or with complex cytogenetic abnormalities treated with 5-azacytidine. (ClinicalTrials.gov NCT00915785). *Methods.* A total of 40 patients were enrolled in this study and received 5-azacytidine 75 mg/m^2 per day subcutaneously for 7 days with each cycle to be repeated every 28 days. Informed consents were obtained from all patients. Clinical, haematological and cytogenetic assessments were recorded and analyzed. Response criteria were based on the modified IWG response criteria for MDS. Seven of these patients received only 1 cycle of treatment and were then excluded from the final analysis. Six patients proceeded to allogeneic stem cell transplantation and their data were censored at the time of transplant. *Results.* The median age of the cohort (n=40) was 64 (range 17-82). Based on WHO classification, 1 patient had RARS, 7 had RCMD, 26 had RAEB I/II and 6 had acute myeloid leukaemia transformed from MDS. The IPSS of the cohort were Intermediate-2 (n=27) and high risk (n=13). Twelve patients had isolated chromosome 7 anomalies (including monosomy 7 and deletion of 7q) and 28 had chromosome 7 abnormalities as part of a more complex cytogenetic profile. Median number of treatment cycles delivered was 6 (range 1-21). Based on the modified IWG response criteria, 4 patients achieved complete response (CR), 6 had partial response (PR) and a further 4 had haematological improvement (HI). The overall response rate (ORR=CR+PR+HI) was 42% (5/12) for isolated chromosome 7 anomalies and 32% (9/28) for chromosome 7 with additional cytogenetic abnormalities. Complete cytogenetic response was observed in 5 patients and partial cytogenetic response in another 8 patients. The median overall survival (OS) was 10.5 months (n=33). There was no significant difference in survival between the patients with isolated chromosome 7 anomalies and those with more than one cytogenetic abnormalities (median OS: 10.5 vs 9.7 months, P=0.8). The ORR were 50% (5/10) and 39% (9/23) for these 2 groups respectively. The median OS were 12.1 months for responders and 7.9 months for non-responders (P=0.05). Total 13 patients achieved cytogenetic response and their median OS was 14.1 months compared with 8.2 months for those without any cytogenetic response (P=0.01). *Conclusions.* The interim results of this study indicate that the use of 5-azacytidine is associated with a high overall response and cytogenetic response in MDS patients with chromosome 7 abnormalities. Significantly, attainment of overall response or cytogenetic response correlates with an improved OS.

0325

HIGH FREQUENCY OF THE NQO1 C609T HOMOZYGOUS MUTANT GENOTYPE IN PATIENTS WITH MYELODYSPLASTIC SYNDROME AND ISOLATED TRISOMY 8

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Background. Models for the pathogenesis of myelodysplastic syndromes (MDS) imply the role of cumulative genetic and xenobiotic factors in genetically predisposed individuals. The widely expressed NAD(P)H:quinone oxidoreductase 1 (NQO1) enzyme is involved in the cellular response to oxidative damage protecting cells from quinones, the ultimate toxic metabolites after exposure to benzene. The encoding gene is subject to the genetic single nucleotide polymorphism C609T, changing the aminoacid sequence (Pro187Ser), resulting in enzymatic inactivation. Individuals homozygous for the mutant allele (T/T) completely lack NQO1 activity, whereas heterozygotes

(C/T) present threefold decreased enzymatic activity. We hypothesized that the *NQO1* gene polymorphism may predispose individuals to a greater risk of MDS and/or promote specific types of chromosome aberrations in myeloid malignancies. *Aims.* To investigate the potential role of NQO1 C609T inborn polymorphism in MDS pathogenesis, we performed a case-control study analyzing the *NQO1* genotypic distribution in a large group of Greek patients with primary MDS. We next compared the genotypic frequencies in patients with isolated +8, del(5q) and -7/del(7q), since these represent the commonest unbalanced aberrations in MDS and may indicate prior exposure to xenobiotics. *Methods.* The NQO1 C609T polymorphism was investigated in 261 MDS patients and 270 matched healthy controls using a PCR-RFLP assay. It was evaluated in respect to patient characteristics, chromosome abnormalities and IPSS classification. The *NQO1* genotype was additionally investigated in 66 MDS patients carrying the recurrent unbalanced abnormalities +8, del(5q) and -7/del(7q) as sole abnormalities. *Results.* A cytogenetic result was achieved in 255 out of 261 patients at diagnosis. Among them, 103 (40.4%) showed clonal karyotypic abnormalities. The most frequently observed single abnormality was trisomy 8 (25/255, 9.8%). The distribution of the *NQO1* genotypes did not differ significantly between MDS patients and controls (homozygous wild type (C/C) 65.1 vs 61.2%; heterozygotes (C/T) 33 vs 37%; homozygous mutant (T/T) 1.9 vs 1.8%). Stratification of patients according to gender, age, cytogenetic and IPSS groups revealed no differences in the mutant (C/T and T/T) genotypic frequencies. To investigate the impact of *NQO1* polymorphism in the pathogenesis of certain recurrent karyotypic imbalances, we additionally analyzed 66 MDS patients with +8 (n=30), del(5q) (n=29) and -7/del(7q) (n=7) as sole abnormalities. We observed no differences in the frequencies of NQO1 polymorphism between patients with del(5q) and healthy controls. On the contrary, the NQO1 polymorphism was associated to an increased risk of MDS with isolated chromosome 7 abnormalities (P=0.033). Moreover, an increased frequency of the homozygous mutant genotype (T/T) was observed in patients with isolated trisomy 8, as compared to the controls (10% vs 1.8%, P=0.032). *Summary/Conclusions.* Our results show that the *NQO1* C609T polymorphism does not correlate with susceptibility to MDS. The high incidence of NQO1 mutant genotype in patients with -7/del(7q) is noteworthy, since chromosome 7 aberrations in primary MDS are indicators of previous exposure to benzene. The high frequency of the homozygous mutant genotype in our patients with isolated trisomy 8, reflecting lack of NQO1 enzyme activity, might confer a subgroup with defined prognostic significance within the heterogeneous population of +8 patients.

0326

COSTEFFECTIVENESS OF AZACITIDINE FOR THE TREATMENT OF IPSS INT-2 OR HIGH-RISK MYELODYSPLASTIC SYNDROMES IN THE UK

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Introduction: Myelodysplastic syndromes (MDS) are fatal, progressing hematological malignancies affecting approximately 1 in 30,000 people in the UK. There is considerable unmet treatment need in patients, owing to the high morbidity, transfusion burden, risk of progression to acute myeloid leukemia (AML) and high mortality. A recently published (Fenaux *et al*, 2009) phase III, randomized, international, parallel-group, open-label trial (AZA-001) in IPSS Int-2 or High-risk patients with MDS showed a 2-fold increase in 2-year overall survival (OS) with azacitidine (AZA, 51%) compared with conventional care regimens (CCR, 26%), which comprised low-dose cytarabine (LDAC), intensive chemotherapy (IC) and best supportive care (BSC). AZA also was associated with a significantly reduced risk of progression to AML (HR: 0.50, 95% CI: 0.35-0.70) and a significant reduction in transfusion dependence compared with CCR. AZA is a cytidine nucleoside analog that appears to exert disease-modifying effects in MDS. *Aim.* The aim of this analysis was to evaluate the incremental cost-effectiveness ratio (ICER) of AZA compared with combined CCR and with LDAC, IC or BSC individually in the UK. *Methods.* A lifetime Markov cohort-based decision-analytic model was developed to examine the effect of treatment on patients with IPSS Int-2 or High-risk MDS. OS rates were extrapolated from the AZA-001 study. Health utility scores were mapped from the European Organisation for Research and Treatment of Cancer (EORTC) quality of life (QoL) scores collected during the Cancer and Leukemia Group B (CALGB) study 9221 reported elsewhere (Silverman *et al,* 2002). Resource usage was estimated from a survey of UK hematologists. The ICER assessed the incremental cost per quality-adjusted life-year (QALY) gained. A probabilistic sensitivity analysis using Monte Carlo simulation was used to evaluate uncertainty of the parameters included in the model. The analysis also includes the recent methodological guidance that has been issued by the National Institute for Health and Clinical Excellence (NICE) in the UK regarding the assessment of certain life-extending medicines. *Results.* The results show that AZA provides a significant incremental OS gain of more than 2 years versus CCR, and patients also have an improved QoL demonstrated by QALY gains of 1.68 compared with BSC, 1.83 compared with LDAC and 1.84 compared with IC. These results lead to an ICER of £37,105 per QALY compared with IC, £40,754 compared with LDAC, and £47,432 compared with BSC. Given the poor prognosis for patients with IPSS Int-2 or High-risk MDS and the ability of AZA to significantly extend OS in this population, the application of end-of-life valuation methodology as guided by NICE reduces the ICERs to £26,504 per QALY compared with IC, £29,110 compared with LDAC and £33,880 compared with BSC. Sensitivity analyses suggest that the cost-effectiveness results are significantly influenced by the choice of the parametric curve fit used to extrapolate OS, and the health utility scores. *Conclusions.* Compared with combined CCR, AZA offers an innovative, cost-effective treatment option for patients with IPSS Int-2 or High-risk MDS in the UK. Treatment with AZA significantly increases QoL in a disease with a poor prognosis and with associated morbidity.

0327

ASSESSMENT OF HEALTH-RELATED QUALITY OF LIFE AT DIAGNOSIS IN AN EUROPEAN REGISTRY FOR LOW RISK AND INTERMEDIATE-1 RISK MDS: REPORT ON THE FIRST 322 PATIENTS

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Background. To describe the demographics, the disease-management and the clinical outcome in Myelodysplastic Syndromes (MDS) a prospective, multicenter European Registry (EUMDS) for newly diagnosed IPSS low and intermediate-1 patients has been initiated under the auspices of the European LeukemiaNet. *Aims.* As data on health-related quality of life (QoL) in MDS patients are rare, the EQ-5D (European quality group 5 dimensions) descriptive system was introduced in the EUMDS evaluation. The performance status was assessed by the Karnofsky score and comorbidities at diagnosis were assessed by the Sorror index. *Methods*. In 322 out of 400 EUMDS patients analyzed so far the EQ-5D score has been applied at initial presentation. *Results.* The median age was 74 yrs (female 75.2; male 73.5) with 203 (63%) male patients. Comorbidities were detected in a substantial proportion of patients (mean Sorror score 2.4, range (0 to 11). Whereas a reduced performance status as defined by a Karnofsky Index £ 70 was detected only in 20% of MDS patients, an impaired QoL was observed in a greater proportion of patients. Using EQ-5D the dimensions mobility, self-care, usual activities and pain/discomfort were clearly age-dependent. No problem (Level 1) was detected in 80, 96, 50 and 58% of 50-59 years old persons, whereas the cohort of 70-79 years old revealed a lower percentage of 55, 80, 66 and 51% respectively. Similarly the self-reported health as assessed by a visual analogue scale (VAS) was lower in elderly patients; mean score of 74 (standard deviation (sd) 19.0) in 50-59 yrs, 73 (21.3) in 60-69 yrs, 66 (22.2) in 70-79 yrs and 62 (20.3) in 80+ yrs. Moreover, analyses revealed pronounced sex differences in distinct dimensions as no problem (Level 1) was detected in 58% male and 45% female patients in the dimensions mobility (P<0.05), in usual activities 70 vs 54% (P<0.001), in pain/discomfort 56 vs 41% (P<0.05) and in anxiety/depression 69 vs 45% (P<0.001). In contrast this effect was less pronounced in the dimension self-care as Level 1 was detected in 86% of male and 80% of female patients (ns). The EQ-5D status was reported in 10 different countries contributing to this registry. The mean

EQ- 5D VAS score was 68 (sd 21.9) and ranged from 52 (sd 16.4) in Austria to 84 (13.1) in Greece. This difference might be explained by intercountry, cross-cultural differences or differences in patient recruitment by country. *Summary/Conclusions*. This is the first prospective analysis of health-status and QoL in a large cohort of newly-diagnosed MDS patients. Relevant restrictions in self-reported health are shown in MDS patients. In the evaluation of QoL in MDS age- and gender effects as well as possible cross-cultural differences should be considered and integrated. EQ-5D value sets, representing the general population from European countries will allow comparisons to be made between the general population and patients with MDS and will contribute to understand the impact of MDS on QoL. EQ-5D will be prospectively reassessed 6-monthly in all patients continuing follow up in the registry.

0328

RED BLOOD CELL (RBC) ALLOIMMUNIZATION AFTER BLOOD TRANSFUSION IN PATIENTS WITH MYELODYSPLASTIC SYNDROME (MDS) OR CHRONIC MYELOMONOCYTIC LEUKEMIA (CMML)

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Background. RBC alloimmunization is a major problem in chronically transfused patients because it exposes the blood recipient to the risk of delayed hemolytic reactions and makes it difficult to find compatible blood. *Aim.* To determine the incidence and characteristics of RBC antibodies in chronically transfused patients with MDS or CMML. *Material and Methods.* We reviewed the transfusion and clinical records of 337 patients with MDS or CMML seen at our hospital from 1990 to 2009. Patients were included if they had received ≥2 RBC transfusions, at least one month had elapsed from first to last transfusion, and no RBC antibody was detected at first transfusion. Testing for RBC antibodies was performed prior to each transfusion. For the purpose of this study only RBC antibodies of IgG class, usually of immune nature, were taken into account. Complex immunization was defined as the appearance of free autoantibody in serum or a combination of alloantibodies that decreased the probability of finding compatible blood below 3%. The cumulative incidence of RBC immunization was calculated by taking death as a competing risk. Incidence rates were compared by Poisson multivariate regression. *Results.* 272 patients met the eligibility criteria. Median age was 75 (16-99) years and 149 (55%) were males. The most frequent diagnoses were refractory cytopenia with multiliniage dysplasia (RCMD, n=85), type 2 refractory anemia with excess blasts-2 (RAEB-2, n=45), type 1 chronic myelomonocytic leukemia (CMML-1, n=37), refractory cytopenia with multiliniage dysplasia and ringed sideroblasts (RCMD-RS, n=30), and other types of MDS (n=75). Median follow-up from first transfusion was 1.4 (range: 0.1-19) years, and the median number of RBC units transfused per patient was 33 (range: 4-330). Forty-five patients formed 81 alloantibodies and 10 autoantibodies, and 11 evolved into a complex immunization. Twenty-two patients formed one antibody, 11 formed two, and 12 formed three or more antibodies. Anti-K1 and anti-E were the most frequent individual specificities (26 and 19, respectively), followed by anti-c (6) and anti-Jka (5). In 26 (59%) patients, all the alloantibodies they formed were directed against the K1 or antigens of the Rh system. In 7 out of the 10 cases with autoantibody, the latter was detected in patients already alloimmunized. The incidence rate of antibody formation was 1 per 10.5 person-years of follow-up and was not influenced by sex, age or cytologic diagnosis after adjustment for the number of transfusions. The cumulative incidence of presenting at least one antibody increased with the number of RBC transfusions; it was 14% (95%CI: 10%-19%) after 28 RBC units and reached a plateau at 19.5% (95%CI: 14%-23%) after 130 RBC units. At that time, the cumulative incidence of complex immunization was 6%. *Conclusions.* Patients with MDS and CMML exhibit a high rate of RBC immunization, which appears soon after starting on chronic transfusion support and is mostly directed against the K1 and antigens of the Rh system. It is conceivable, therefore, that transfusing these patients with extended compatibility, including the K1 and CcEe antigens, would yield a substantial reduction of the RBC alloimmunization rate.

Myeloma and other monoclonal gammopathies - Biology 1

0329

FISH AND IMMUNOPHENOTYPE IN ELDERLY UNTREATED MULTIPLE MYELOMA PATIENTS ENTERED IN A PROSPECTIVE RANDOMIZED TRIAL OF VELCADE-MELPHALAN-PREDNISONE AND THALIDOMIDE VS VELCADE-MELPHALAN-PREDNISONE

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Background. Recurrent chromosomal abnormalities have been identified in Multiple Myeloma (MM) and these have been related to clinical course and response to therapy. Moreover, the clinical and prognostic value of immunophenotyping in MM have been demonstrated by many studies. *Aims.* To evaluate chromosomal abnormalities and immunophenotype in a large series of elderly MM patients and the correlation between different parameters in order to identify new prognostic characteristics related to response to therapy. *Methods.* Between May, 2005 and January, 2009, 511 patients aged ≥ 65 years were randomized to receive VMPT-VT (N=254) or VMP (N=257). In 336 patients FISH analysis was performed on bone marrow plasma cells (BMPC) purified using anti-CD138-coated magnetic beads. Nuclei from fixed PC were prepared for interphase FISH using standard *Methods.* DNA probes were used to detect 13q14, 12p13, 1p36 and 17p13.1 deletions; t(4;14)(p16;q32), t(14;16)(q32;q23), t(11;14)(q13;q32); 11q23 (MLL) and 1qter gains. Ploidy status was analyzed by chromosome 9,11,15 enumeration. The immunological phenotype of BMPC was assessed in 399 patients using triple or quadruple combinations of MoAbs for the detection of the following antigens: CD38, CD138, CD56, CD45, CD40, CD19, CD20, CD52, CD117, kappa/lambda. *Results.* In 90% of all patients at least one chromosomal abnormality was found. Del13 was identified in 53.9% of all patients. A significant correlation was observed between del13 and higher levels of LDH (P=0.009) and beta2-microglobulin (P=0.03), lower levels of Hgb (P=0.002) and female gender (P=0.02). Moreover, BMPC with del13 were more frequently CD45 and CD19 negative (P=0.003 and P=0.006, respectively) and correlate with delp53 and t(4;14)(P=0.005 and P=0.0002, respectively). Delp53 was found in 16.4% of all patients and no significant correlation was found according to clinical and phenotypic parameters. 18.9% of all patients showed t(4;14) and a significant correlation was found with t(14;16) (P=0.00003) and lower levels of CD117 (P=0.00001). t(14;16) was detected in 9.8% of 173 patients and showed lower expression of CD45 e CD19 (P=0.01 and P=0.02, respectively).

Table 1.

	VMPT-VT			VMP		
%	CR+VGPR	No CR+VGPR	*P* Value	CR+VGPR	No CR+VGPR	*P* Value
CD20 mean expression	8.8	16.3	0.007	10.3	18.4	0.01
CD117mean expression	17.4	25.2	0.04			ns
CD45 mean expression			ns	9.2	15.2	0.02
CD19 mean expression			ns	18.6	26.7	0.01
t(11;14)+ patients	8.6	27	0.001			ns
non-hyperdiploid patients	51.3	81.8	0.02			ns

The presence of t(11;14) was found in 16.2% of all patients and has been associated with lower levels of CRP (P=0.0002) and higher expression of CD20 (P=0.000001). Deletion of 12p13 and 1p36 was identified in 15.6% and 20.6% of 109 and 50 patients, respectively. Gain of 11q23

e 1q21 was found in 57% and 42% of 159 and 50 patients, respectively. Ploidy status was analyzed in 105 patients and 59.1% showed a non-hyperdiploid status. Significantly higher expression of CD20 was identified in patients not achieving CR+VGPR, in both VMPT-VT and VMP groups. Other phenotypic and cytogenetic features significantly correlating with response to therapy are shown in Table1. *Conclusions.* Our results indicate that: 1- Higher expression of CD20 negatively influences the response to therapy in both arms. 2- Higher expression of CD117, higher frequency of t(11;14) and non-hyperdiploid status correlate with absence of CR+VGPR only in VMPT-VT arm. 3- Higher expression of CD45 and CD19 correlates with absence of CR+VGPR only in VMP arm.

0330

ANALYSIS OF CIRCULATING MICRORNAS EXPRESSION PROFILE IN PATIENTS AFFECTED BY MULTIPLE MYELOMA AND MGUS

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Background. Recent studies of microRNA (miRNA) expression profile performed on neoplastic plasmacells have demonstrated that miRNAs are involved in Multiple Myeloma (MM) pathogenesis. However to date, little is known about the role of circulating miRNAs in this malignancy. It has been shown that miRNAs circulate in a stable cell-free form in the blood-stream so that they can serve as ideal biomarkers for cancer. *Aim of the study*. To investigate the expression of miRNAs in the plasma of newly-diagnosed Myeloma patients and to detect a characteristic circulating miRNA signature to use for disease monitoring. *Methods*. We have developed a method for isolating miRNAs from blood plasma by modifying mirVana™ miRNA Isolation Kit (Ambion Inc). MicroRNAs have been isolated from peripheral blood (PB) plasma as well as from bone marrow (BM) blood plasma and CD138+ malignant plasmacells. The miRNAs expression profile has been examined using a quantitative PCR-method (TaqMan® Human microRNA cards, Applied Biosystems) that allows the analysis of 365 human miRNAs by low density array technology. Plasma samples of normal subjects have been included in the study. Relative quantification of miRNA expression has been calculated with the 2-ΔΔCt method. The data have been normalized respect to MammU6 and relative to a calibrator sample (average of normal subjects plasma samples). Differentially expressed miRNAs have been identified using "Significant Analysis of Microarrays"(SAM) algorithm, the t test and the nonparametric Wilcoxon rank sum test. The data have been analysed comparing the results with the $CD138^+$ malignant plasmacells miRNA signature reported in the literature. *Results.* We have analyzed the plasma of 10 healthy donors, 5 MGUS patients and 15 newly diagnosed myeloma patients. The miRNA expression profile observed in the peripheral blood plasma faithfully traces that of the bone marrow plasma and that of $CD138^+$ isolated plasmacells. The comparison of the miRNA expression profiles revealed a group of 47 miRNAs that are overexpressed in the plasma of patients versus healthy donors. Among these, a group of 9 miRNAs are upregulated in the plasma of both MGUS and myeloma patients, with a higher expression in the latter group. Six circulating miRNAs are specifically upregulated in MGUS patients whereas the remaining 32 miRNAs are found at high levels only in the plasma of myeloma patients but not in the MGUS group. Seven of the upregulated miRNAs detected in our study have been previously seen deregulated also in the plasmacells1. Among them, hsa-miR-191 that targets CDK6 and hsa-miR-197 that targets the tumor suppressor FUS1, are highly upregulated in the plasma of myeloma patients ($P<0.001$ and $P=0.05$ respectively). *Conclusions.* Specific miRNAs can be detected and quantified in the plasma of myeloma patients. Although very preliminary, these data suggest that the levels of differentially expressed miRNAs can significantly distinguish healthy donors from patients and MGUS from myeloma patients. This analysis could potentially represent a method to assess response to therapy in myeloma disease.

0331

MULTIPLE MYELOMA MMUNOGLOBULIN SEQUENCES SHOW NO INTRA-DISEASE CLUSTERING BUT ARE OCCASIONALLY RELATED TO REPERTOIRES FROM NORMAL LYMPHOCYTES AND OTHER B-CELL TUMORS

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Background. The characterization of stereotyped immunoglobulin receptors has improved our knowledge on the antigen-driven pathogenesis of several lymphoid tumors, including chronic lymphocitic leukemia (CLL), marginal-zone lymphoma (MZL) and mantle-cell lymphoma (MCL). Multiple myeloma (MM) is a post-germinal center neoplasm no longer expressing membrane-bound immunoglobulins; however antigen stimulation might have played a role during early disease phases. Immunoglobulin heavy chain (IGH) genes have not been extensively investigated in MM, because of lack of large sequence databases. *Aims.* To address this issue, we created a database of MM sequences including our institutional records as well as sequences available from the literature. We planned a two-step analysis, characterizing first the MM repertoire and performing intra-MM clustering analysis; then comparing the MM series to a large public database of IGH sequences from neoplastic and non-neoplastic B-cells. *Patients and Methods.* 131 MM IGH genes were amplified and sequenced at our Institutions, as described (Voena *et al*., Leukemia 1997). 214 MM IGH sequences were derived from published databases (NCBI-EMBL-IMGT/LIGM-DB) for a total of 345 fully interpretable MM sequences. 28590 IGH sequences from other malignant and non-malignant B-cells were retrieved from the same public databases. All the sequences were analyzed using the IMGT database and tools (Lefranc *et al.*, Nucleic Acid Res. 2005; http://imgt.cines.fr/) to identify IGHV-D-J gene usage, to assess the somatic hypermutation (SHM) rate and to identify HCDR3. HCDR3 aminoacidic sequences were aligned together using the ClustalX 2.0 software (Larkin *et al*., Bioinformatics, 2007; http://www.clustal.org/). Subsets of stereotyped IGH receptors were defined according to Messmer *et al.* (J Exp Med., 2004) and Stamatopoulos et al. (Blood, 2007). *Results.* IGHV-D-J usage and HCDR3 lenght in MM was more in keeping with the normal B-cell repertoire compared to other lymphoid tumors, with only modest over-representation of IGHV3-9, IGHV3-21, IGHV5-51 genes and under-representation of the IGHV3-23 and IGHV4-34; 98% of MM sequences showed a SHM rate >2% with one single patient sharing 100% identity to germline. Intra-MM search for HCDR3 similarity never met minimal requirements for stereotyped receptors. When MM sequences were compared to the public database, only a minority of sequences (2.9%) clustered with those from lymphoid tumors and normal B-cells (Table 1).

Table 1.

Table 1: Mixed clusters of immunoglobulin sequences between multiple myeloma (MM) and normal or neoplastic B-cells

Source of immunoglobulin sequence	Number of sequences analyzed	Number of MM clustering with other sequences	% of present MM database clustering with other sequences	MM: probability of clustering with other sequences
Non neoplastic B-cell	24085	3 (3*)	0.9%	1.2×10^{-4}
Chronic lymphocitic leukemia	3185	5 (3*)	1.5%	1.6×10^{-3}
Marginal-zone lymphoma	195	1 (1*)	0.3%	5.1×10^{-3}
Mantle-cell lymphoma	104	0	0%	0

* Newly identified provisional clusters

Specifically two MM sequences could be assigned to previously identified CLL subsets (n.37 and n.71 according to Murray *et al*., Blood 2008). In addition, three mixed MM/CLL and one MM/MZL provisional clusters were identified. Finally three provisional clusters were found between MM and IGH sequences from normal B-cells. *Conclusions.* The

analysis of the largest currently available database of MM IGH sequences indicate the following: 1) MM IGH repertoire follows a nearly physiological distribution; 2) MM specific HCDR3 clusters do not occur to a frequency detectable with currently available databases; 3) 98% of MM sequences are not related to other "highly-clustered"lymphoproliferative disorders; 4) Occasional clustering of MM with immunoglobulin receptors from normal and (at a slightly higher frequency) neoplastic lymphocytes was noticed. In conclusion, our analysis does not support a critical role for antigen selection in the majority of MM patients.

0332

EXPRESSION OF MET MRNA AS PROGNOSTIC MARKER IN MULTIPLE MYELOMA PATIENTS TREATED WITH NOVEL DRUGS

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Background. MET is the tyrosine kinase receptor for Hepatocyte Growth Factor (HGF). Its aberrant activity has been reported in several tumors including multiple myeloma (MM) where bone marrow (BM) microenvironment produces HGF to stimulate plasma cells growth and migration. In MM cell lines, the inhibition of this pathway causes growth arrest and cell death. *Aim.* to investigate the role of MET mRNA expression as predictor of response and as prognostic marker in MM patients treated with novel agents. Patients and *Methods.* eighty four newly diagnosed MM patients have been evaluated for MET mRNA expression and HGF serum level. Fifty one patients received the PAD-MEL100-LP-L regimen (Palumbo A, JCO 2010) and 33 received nine VMP courses (Palumbo A, 2009 ASH Meeting, abs 128). On BM CD138+ cells harvested at diagnosis, MET mRNA expression has been evaluated using a quantitative Real-Time PCR. Relative quantification of MET mRNA was performed using the ΔΔCt approach. JUM2 cell line was utilized as calibrator and Gus as housekeeping gene. The cut-off value of MET mRNA was selected according to ROC analysis on samples of patients treated with PAD-MEL100-LP-L schedule (learning series) and then validated on samples of patients treated with VMP (validation series). HGF serum value has been determined using ELISA assay. *Results.* Eighty four patients (39 female/45 male) with a median age of 69 yr (range 46-86) have been evaluated. No differences in baseline albumin, β2 μg, BM plasma cell infiltration and cytogenetic abnormalities have been observed between patients with high and low MET mRNA levels.

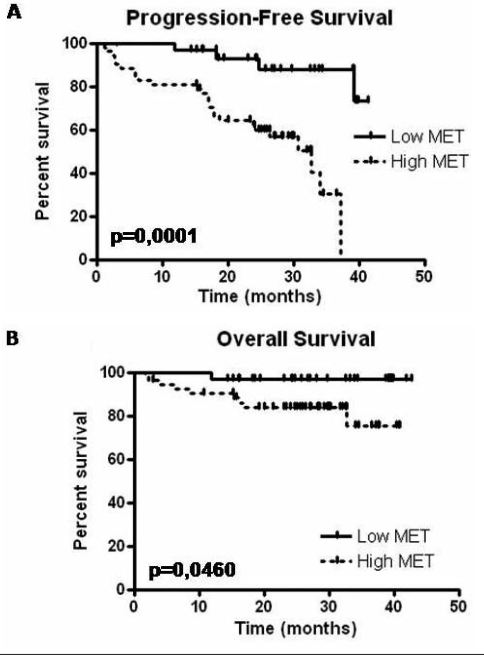

Figure 1.

Considering PAD-MEL100-LP-L patients, MET mRNA expression was significantly higher in those obtaining partial response (PR) or less, compared to patients achieving at least very good partial response (VGPR). In patients enrolled in the PAD-MEL100-LP-L trial this difference was observed after PAD-induction (median MET value 94.1 range 9.7-586.1 vs median 37.92 range 1.1-170.0, P=0.001) and was maintained after autologous bone marrow transplantation (ABMT) (median 148.6 range 27.1-586.1 vs median 65.8 range 11-466.3, P=0.007). In patients treated with VMP, MET mRNA level allowed to identify patients with differences in response rate although without reaching statistical significance. On the whole population of 84 patients, after a median follow-up of 27.7 months, the 2-year PFS in the low and in the high MET mRNA groups was 92% and 62% respectively (P=0.0001) (Figure 1A). The 2-year OS was 96.6% and 83,8% respectively (P=0.0460) (Figure 1B). The prognostic value of MET mRNA expression resulted stronger than those of β2 μg or cytogenetic abnormalities. Even a combined score of MET mRNA expression with any other biological parameter resulted less powerful compared to MET mRNA value alone. HGF serum values resulted similar in both optimal and sub-optimal responders and did not predict PFS and OS. *Conclusions.* 1) high MET mRNA expression level identifies a group of patients with suboptimal response and inferior PFS and OS in both ABMT- and non ABMT-based regimen; 2) high MET mRNA expression seems to be a hallmark of more aggressive disease thus MET can be considered a target for anti myeloma therapy.

0333

DEPLETION OF ONCOGENIC RAS ISOFORMS IMPAIRS SURVIVAL OF MULTIPLE MYELOMA (MM) INDEPENDENTLY OF AKT/PKB SIGNALING

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Introduction: Oncogenic Ras is known to activate survival pathways in numerous cancer entities and N- or K-Ras mutations are also frequently found in MM. However, its precise role in MGUS to MM transition and tumor progression is still unclear and development of inhibitory drugs has so far not been successful, either. *Aims.* We were interested in analyzing the function of oncogenic Ras isoforms and their downstream signaling via Akt/PKB, which per se is an important survival pathway for about 50 percent of primary MM samples. *Methods.* We used shRNA expression constructs against N- or K-Ras to specifically knock down the respective Ras isoform in two N-Ras mutated, one K-Ras mutated, and two Ras wildtype MM cell lines. To control isoform specificity, we tested the shRNA effects on ectopically expressed HA-tagged Ras proteins. Transfection was performed by electroporation and subsequent enrichment through cell sorting. Signaling pathways were analyzed with Western blotting. Flow cytometry was used to measure the rate of apoptotic cells staining with Annexin-V-APC and propidium iodide. A series of different MM patient samples (n=35) were analyzed for Ras mutations by RT-PCR and were submitted to inhibition of Akt/PKB with the small molecule inhibitor Akti-1,2. Where corresponding bone marrow biopsies were available, they were screened for the presence of phosphorylated Akt by fluorescence immunohistochemistry (IHC). *Results.* Isoform-specific Ras knockdown impaired survival selectively in MM cell lines with the respective oncogenic isoform, but not in Ras wildtype cell lines. In particular, survival of the Ras wildtype AMO-1 and U266 cells remained unaffected by Ras depletion, while N-Ras knockdown induced apoptosis selectively in N-Ras mutated INA-6 and JJN-3 cells. Conversely, K-Ras mutated MM.1s cells were most responsive to knockdown of K-Ras. Phosphorylation of Akt (Ser473) was not decreased in Western blots, however. In addition, Ras mutation status in the tested primary MM samples (40% N- or K-Ras mutated) did not correlate with the presence of phosphorylated Akt as shown by IHC (60% positive), or with the sensitivity to Akti-1,2 (49% sensitive), all of which indicates that oncogenic Ras may not signal via the Akt/PKB pathway. Combined shRNA-mediated depletion of both oncogenic Ras and Akt significantly reduced the level of viability. *Conclusions.* Even though pharmacological inhibition of oncogenic Ras is currently not feasible, we show that specifically targeting oncogenic Ras isoforms impairs MM cell survival. This effect does not appear to be mediated through the Akt/PKB pathway, and constitutive Akt/PKB activation in primary MM does not appear to be a consequence of oncogenic Ras. Thus, finding ways to block oncogenic Ras function remains a therapeutically relevant scientific challenge, even more so because the combination with Akt blockade may increase the anti-myeloma effect.

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CENTROSOME AMPLIFICATION AS A POSSIBLE MARKER OF MYELOMA CELL PRECURSORS

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Background Multiple myeloma (MM) is a lymphoproliferative disease characterized by the clonal expansion of neoplastic plasma cells within the bone marrow. Although PCs are recognized as fully malignant cells, the exact developmental stage at which malignant transformation occurs is still unknown. Several candidates for MM clonogenic population have been proposed; however, it is controversial whether clonogenic population is represented by B-cells or PCs, since majority of plasma cells are quiescent. In our study, centrosome amplification (CA) was chosen as potential precursor marker, reflecting mitotic disruption in carcinogenesis. *Aims.* The objective of our study was to evaluate the presence of CA in two populations of B-cell lineage - including B-cells and PCs - from MM patients and to assess whether it is associated with established prognostic factors, such as the most common chromosomal abnormalities in malignant PCs. *Methods*. Immunofluorescent labeling was used for the evaluation of centrosome amplification (CA) in B-cells ($CD19^+$) and PCs ($CD138^+$) of MM patients. Centrin (centrosome protein) copy numbers were used to define three cellular subpopulations: (1) no centrin signal (Non-CS), (2) 1-4 centrin signals (1-4CS) or (3) more than 4 signals of centrin (CA). Samples with ≥11% of B-cells or ≥10% of PCs with >4 fluorescence signals of centrin were considered CA positive. A total of 101 patients were evaluated for CA in PCs and/or B-cells, including 31 patients where both cell types were analyzed. The patient population characteristics were as follows: males/females 50/51, median age of 66 years (range, 40-92 years). Most patients had advanced stage of MM (DS II/III n = 69; ISS II/III n = 61). Interphase FISH with cytoplasmic immunoglobulin light chain staining (cIg FISH) was performed on plasma cells by well-known methods. *Results.* In our previous study, we showed the presence of centrosome amplification in B-cells of MM patients. The frequency of MM cases positive for CA was 34% (26/77) and 34% (18/53) based the analysis of PC samples and B-cell samples, respectively. Overall, 19% (6/31) of MM patients were double-positive. No significant correlation was identified between amount of CA positive PCs and B-cells ($p>0.05$). After splitting patients based on CA threshold, significant correlation was identified only in double-positive group ($r=-0,986$, $P<0.05$). Significant correlation of centrosome amplification in PCs with del(13)(q14), del(17)(p13), gain(1)(q21), or hyperdiploidy wasn't shown. But association of CA in B-cells with PCs hyperdiploidy using 4-point correlation was proven ($\phi=0.45$, $P<0.05$). *Conclusion.* In our study, we showed association of CA in B-cells with PCs hyperdiploidy. This fact relates to role of mitotic disruption in MM aneuploidy and cell carcinogenesis. In association with correlation between B-cells and PCs with CA, we suppose that B-cells with CA are part of myeloma genesis.

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RESPONSE TO VELCADE-THALIDOMIDE-DEXAMETHASONE (VTD) AS INDUCTION TERAPHY IN MULTIPLE MYELOMA PATIENTS CARRYING T(4;14): ANALYSIS OF GENE EXPRESSION PROFILE

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Background. The recurrent immunoglobulin translocation t(4;14)(p16;q32) occurs in less than 20% of Multiple Myeloma (MM) patients and has been frequently associated with poor prognosis in patients treated either with conventional or high dose chemotherapy. The introduction of novel drugs, such as Thalidomide, Lenalidomide and Bortezomib, has been suggested to overcome the negative prognostic impact of high risk cytogenetic abnormalities; in particular Bortezomib has been shown to revert the negative role of the t(4,14) in MM patients. Moreover, clinical studies have shown highly heterogeneous clinical outcome in patients carrying the t(4,14), suggesting that specific biological features might define subgroups of t(4,14) positive patients. *Aim.* In the present study, newly diagnosed MM patients were analyzed by means of gene expression profiling and array-CGH experiments, in order to investigate the molecular mechanisms underlying the response to therapy in the presence or in the absence of t(4;14). *Methods.* The study was performed on a cohort of 50 patients randomly assigned to receive VTD as induction therapy in preparation for ASCT. The presence of t(4;14), del(13) and del(17) was characterize at diagnosis by FISH and/or PCR. In addition, the differential gene expression profile of CD138+ enriched plasma cells was evaluated by means of expression microarray and SNPs array using the Affymetrix platform. Expression data were analyzed by MetaCore® software and significant expression results were validated by Real-time PCR. *Results.* Eighteen out of the 50 analyzed patients carried t(4;14). Both in the positive and negative t(4;14) subgroup of patients, we didn't observe any significant clustering of gene expression based on the presence of the common chromosomal aberrations. On an intention-to-treat basis, 50% of the t(4;14) positive patients and 31% of the t(4;14) negative patients achieved at least a near CR. In both subgroups we compared the lists of genes differentially expressed in responders (R, those who achieved ≤CR or near CR) versus non responders patients (NR, those who achieved >near CR); we observed a differential expression of 2968 and 2136 genes in t(4;14) positive and t(4;14) negative patients, respectively. Moreover in R patients carrying t(4;14), a subset of genes involved in cell adhesion process (e.g. *VE-cadherin*) was significantly up-regulated, whereas the Wnt non-canonical signalling cascade resulted the pathway most affected as a consequence of the significantly down regulation of expressed genes such as *WNT10A* and *FZD7*. In the subgroup of t(4;14) negative patients, plasma cells trascriptome of R was characterized by the overexpression of genes involved in Wnt canonical (e.g. *WNT3, WNT4*) and Notch signalling pathway (e.g. *NOTCH1, NOTCH2*), and by the down regulation of pro-apoptotic genes (e.g. *CASP10*). *Conclusions.* The differential expression of genes involved in pathways controlling cell development and differentiation suggests a distinct features of the MM clone in R patients carrying t(4;14) compared to NR, and provides new insight into the biological characteristics related to the sensitivity of MM cells to treatment based on new drugs combination

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0336

EVOLUTION FROM MGUS TO MM IS CHARACTERIZED BY A CLONAL EXPANSION OF GENETICALLY ABNORMAL PLASMA CELLS

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Background. Genetic aberrations detected in MM have also been reported in MGUS. However, the distribution of the genetic abnormalities between the asymptomatic precursors of MM, MGUS and smoldering MM (SMM), and the symptomatic MM seems to be different. *Aims.* To determine the frequency of chromosomal abnormalities and the proportion of plasma cells (PC) carrying these ones in three evolution stages of MM (MGUS, SMM and MM). *Methods.* We analyzed 88 patients with MGUS, 88 with SMM and 88 with symptomatic MM. Patients were classified into the three entities according to the IMWG (International Myeloma Working Group) criteria. PCs were purified using anti-CD138 immunobeads in all the bone marrow samples (purity was above 90% in all cases). Genetic abnormalities were evaluated by fluorescence *in situ* hybridization (FISH) with probes for IGH rearrangements, including t(4;14), t(11;14) and t(14;16), 13q14 and 17p13 deletions, and 1q21 gains. Multiparametric flow cytometry (FC) was performed for the characterization of aberrant PCs (CD38-FITC/CD56-PE/CD19-PerCP-Cy5/CD45-APC). In specific cases an additional staining for cytoplasmatic Ig light chains was used to clarify the polyclonal vs monoclonal nature of PCs. *Results.* The incidence of IGH translocations globally considered, t(4;14), 13q deletions and 1q gains was significantly higher in SMM/MM comparing to MGUS: 40/43% vs 25% for IGH translocations, 14/11% vs 4.5% for t(4;14), 39/45% vs 20% for 13q deletions and 42/53% vs 25.5% for 1q gains. In contrast, no significant differences in the frequency of specific chromosomal abnormalities were found between SMM and MM. The proportion of PC carrying any of the genetic abnormalities explored by FISH was increasing progressively from MGUS to SMM and to MM. Thus, the median percentage of PC with t(11;14) and 13q deletion was significantly lower in MGUS than in SMM, and in SMM than in MM status (38% vs 62% vs 84% and 35% vs 56% vs 77% for each abnormality respectively). Differences in the proportion of PC exhibiting the t(4;14) were also found when comparing both premalignant stages (MGUS and SMM) with MM. Furthemore, the median proportion of PC carrying 1q gains was

significantly lower in MGUS than in SMM/MM. In order to know if the increase of PC with genetic abnormalities across the evolution stages of MM was related to the less proportion of normal residual PC in MM compared to MGUS, we related the results derived from the genetic analysis by FISH with the data provided by FC. Even though the proportion of aberrant PC was lower in MGUS compared to MM, only a fraction of clonal PC in MGUS displayed the genetic abnormal clone (Figure 1). We found this same observation in SMM although in this stage the proportion of aberrant PC was similar to MM. *Summary.* Although chromosomal abnormalities recurrently found in MM were also present in MGUS, the incidence of t(4;14), 13q deletions and 1q gains was significantly higher in SMM/MM than in MGUS. Interestingly, the proportion of abnormal PCs displaying the chromosomal abnormalities was significantly increasing from MGUS to SMM and to symptomatic MM.

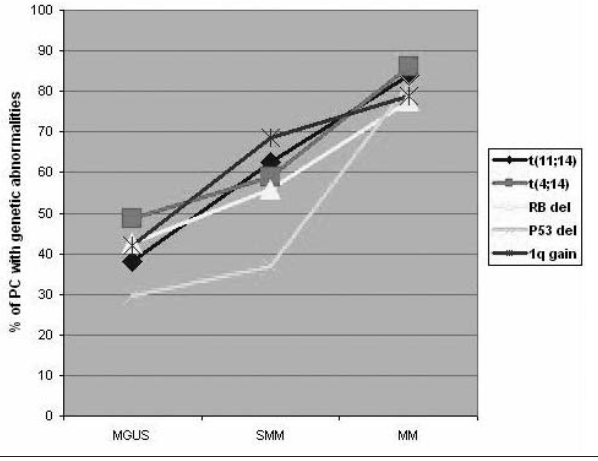

Figure 1. Median percentage of PC with genetic abnormalities.

0337

MICROFIND APPROACH FOR FISH DETECTION OF GENETIC LESIONS FROM SCARCE CELL SAMPLES IN MULTIPLE MYELOMA SAMPLES

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Background. Despite the improvement in sensitivity for the identification of chromosomal aberrations by fluorescence based methods and automated analysis (Ntouroupi *et al.* Br J Cancer. 2008; Medintz *et al.* Int J Nanomedicine. 2008), a low cell number recovery may make it difficult to perform FISH detection of rearrangements involved in cancer cells. Multiple myeloma (MM), a clonal B-cell malignancy characterized by the accumulation of terminally differentiated bone marrow plasma cells (PC), represents a peculiar example because of the low infiltrate of malignant cells found in a large fraction of patients. Therefore, the manipulation and the analytical processing of PCs still represents a technological challenge and FISH detection requires purification which generally provides a limited number of PCs. *Methods.* To overcome the difficulty, we have used a novel device (microFINDTM) and tested it for FISH analyses by collecting living cells of bone marrow aspirates from MM patients. microFIND is a glass slide coated with a nanomaterial (Carbone et al. Biomaterials. 2006) that efficiently immobilizes cells (down to 4x103) inside a microchannel: a miniaturized FISH protocol (using one tenth of reagent employed in the standard protocol) is carried out and the slide evaluated by high resolution fluorescence microscopy. PCs were purified from MM bone marrow samples of 10 different patients using CD138 immunomagnetic microbeads (MidiMACS system) as previously described (Fabris *et al*. Genes Chromosomes Cancer 2005). Genetic lesions such as del(13q14), del(17p13) and the t(4;14)(p16;q32) were evaluated using commercially available kits (Abbott Chicago, IL) *Results.* When FISH results by microFINDTM and standard protocols were compared , we found a complete concordance for all tests in all cases studied. *Conclusions.* mincroFINDTM may represent a promising useful tool for the FISH analysis of genomic alterations in myeloma purified plasma cells even in case of a very limited number of available cells. Furthermore the miniaturization of the assay offers a cost saving approach, suitable for automation and throughput increase in a "chip"configuration.

0338

IMMUNOSTIMULATORY OLIGONUCLEOTIDE METAPHASE INDUCTION IN PATIENTS WITH MULTIPLE MYELOMA: A NEW APPROACH FOR G-BANDING ANALYSIS.

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Background. Cytogenetic abnormalities play an important role as prognostic factors in multiple myeloma (MM). However, classical cytogenetic fail to detect these aberrations because the low mitotic index of plasma cells that reduces the availability of analyzable metaphases. The analysis of a set of subjects for the most commonly known aberrations is usually done by FISH on interphase cell. AIMS The goals of this investigation were the use of the oligonucleotide DSP30 in combination with IL-2 and IL-10, as a B-cell mitogen for cytogenetic investigation in MM and the comparison between the karyotype analyses obtained (G-banding) with FISH profile from unstimulated cells. METHODS For metaphase induction, bone marrow mononuclear cells of 27 patients, being 25 with MM and two with monoclonal gammopathy of undertemined significance (MGUS) were cultured in RPMI 1640 medium with 20% fetal calf serum in the presence of the immunostimulatory CpG-oligonucleotide DSP30 and IL-2/IL-10 for 72h. Additionally, two extra set of cell culture was performed for each patient without any stimulant agent (G-banding analysis, when possible and FISH). The FISH panel included probes for the detection of translocations involving IGH gene (14q32), gains associated to 11q23-q25 and 1q21 and deletion of 13q14. The cut off levels for IGH translocations was (>3.2%), gains of 11q23-q25 and 1q21 (2.4% and 2.7%, respectively) and deletion 13q14 (2.8%). All cut off values were established according to the FISH patterns observed in a group of 6 age and sex-matched normal control peripheral blood samples studied with the same probes. *Results.* In the cells treated with DSP30/IL-2/IL-10 it was observed that among 27 samples studied, the cytogenetic analysis was possible in 22 patients (81.4%). On the other hand, in the group without any stimulus, the cytogenetic profile was successful in 21 patients (51.8%), being 6 (42.8%) with normal karyotype and 8 with chromosomal abnormalities (57.2%). Considering the group that received stimulus, normal karyotype was found in 8 patients (31.8%) and metaphases with abnormal karyotype were seen in 14 subjects (68.2%). Among the cytogenetic profile obtained in both groups were observed aneuploidies involving the chromosomes +3, +5, +9, -13, -14, +15, +16, -18, +19, +20, -22 and structural abnormalities such as add(1)(p21), inv(3)(q21q26), del(13)(q14), add(14)(q32) and t(14;16)(q32;q23). FISH analysis performed in those patients whose bone marrow cells were not stimulated displayed the same chromosomal abnormalities as identified in the group with DSP30/IL-2/IL-10. *Conclusion.* These results indicate that the addition of the immunostimulatory oligonucleotide DSP30 in combination with IL-2 and IL-10 showed to be effective to induce cell cycle progression of MM cells *in vitro*. The limited spectrum of chromosomal abnormalities seen by using FISH analysis, with commercial probes may contribute to underestimate the cytogenetic profile in patients with MM. Thus, this new approach represents an easy and cheap method for metaphase induction in plasma cells.

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0339

ASSOCIATION OF PLASMA CELL PHENOTYPE WITH CYTOGENETIC FINDINGS IN MULTIPLE MYELOMA

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Background. Multiple myeloma (MM) is characterised by presence of malignant plasma cells (PC). These PCs are phenotypically heterogeneous with presence of different cytogenetic changes. As genetic abnormalities are important prognostic indicators, surface expression of some markers could be related to patient's prognosis as well. *Aims.* To find relation between antigenic profile and cytogenetic aberrations in MM patients. *Methods.* Were done in 110 newly diagnosed untreated MM patients with median age 67 years (range 40-88 years). Bone marrow $CD38^+CD138^+$ PCs were analysed for expression of CD19, CD20, CD27, CD28, CD56 and CD117 by immunophenotyping, and PCs were considered positive for given marker when its expression on PCs was higher than 30%. Interphase fluorescent *in situ* hybridization (FISH) on separated PCs was used for analysis of del(13)(q14), del(17)(p13), t(4;14)(p16.3;q32), 1q21 gain and hyperdiploidy. *Results.* Plasma cells were detected in all patients with median 8.4% (range 0.1-75.3). Clonal $CD19^+$ PCs were found in 1 patient with del(13)(q14). Other 4 cases with $CD19^+$ PCs were phenotypically normal residual polyclonal PCs without any chromosomal abnormality. Expression of CD20 was found in 8.2% (9/110) patients and there was no significant correlation with any cytogenetic changes in $CD20^+$ and/or $CD20^-$ samples, although del(13)(q14) was found in 42.6% (43/101) of CD20 negative samples. Markers CD27 and CD28 were expressed in 31.8% (35/110) and 20.9 (23/110) patients, respectively, without differences in cytogenetic profile for CD27 and CD28 positive and/or negative cases. PCs not expressing CD56 were detected in 20.0% (22/110), and similar distribution of cytogenetic changes in CD56 positive and/or negative cases was found. Expression of CD117 was found in 31.8% (35/110) patients and this expression correlated with hyperdiploidy [51.4% vs 26.7%; P<0.05], but on the other hand, negativity for CD117 was associated with del(13)(q14) [50.7% vs 20.0%; P<0.01]. Cytogenetic analysis shown del(17)(p13) and t(4;14)(p16.3;q32) as less frequent aberrancies in MM which are not joined to specific PCs phenotype and also t(4;14) was not found in any of $CD20^+$, $CD117^+$ and CD56- cases. *Summary/Conclusions.* Plasma cells are very heterogeneous in their phenotypic and cytogenetic profiles. Our result shown CD117 (its positivity and/or negativity) as the only marker corresponding to chromosomal abnormalities, but further analysis of patients could bring some new information, especially when immunoglobulin heavy chain (IGH) translocation to other partners will be analysed.

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$CD138^+$ CELL SEPARATION AFFECTS CANCER GENE EXPRESSION IN HUMAN MYELOMA CELL LINES

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Background and Aim. Gene expression profiling (GEP) of multiple myeloma (MM) cells is a promising means of identifying high-risk MM patients.[1-3] The analyses depend on plasma cell purification, usually by $CD138^+$ cell separation. Considering the sensitivity of gene transcription,[4] one might ask if separation distorts true *in vivo* GEP patterns. We performed a controlled study of running human myeloma cell lines (HMCL) through a standard $CD138^+$ separation procedure and comparing the resulting GEP effects. *Methods.* We grew 4 HMCLs under standard conditions: U266, INA-6, RPMI 8226, and NCI H929. We stored sample cells in a 3 mL EDTA-coated test tube and separated them, first by Ficoll® density gradient, then by automated immunomagnetic $CD138^+$ technique (StemCell Technologies). We isolated total RNA using a TRIzol® Reagent-based protocol. Using U266 as a screening model, we performed global GEP analysis using Affymetrix GeneChip® Human Gene 1.0 ST Array. Gene level summaries were extracted using RMA.[6] Differentially expressed genes were calculated using the LIMMA package[7] and Ps adjusted using Benjamini-Hochberg transformation. Sample cells showed significant changes (adj. P<0.05) in 670 genes compared to the non-separated controls. We searched for upregulated genes of myeloma and/or cancer relevance and chose *FOS* [8] (absolute fold change (abs.FC)=10.3, adj.P=0.004), *DUSP1* [9] (abs.FC=9.93, adj.P=0.0001), *NFKBIA*[10] (abs.FC=2.06, adj.P=0.002), *ATF4* [3,11] (abs.FC=1.98, adj.P=0.0007), and MIRN21 (abs.FC=4.21, adj.P=0.003)[12,13] for PCR validation. We used NCBI Primer-Blast for primer design and performed real-time quantitative PCR (qPCR) using SYBR® Green technology. The Ct files were analyzed in SAS version 9.1 on the basis of the ΔΔCt method.[14] *Results.* In all 4 HMCLs FOS and *NFKBIA* were upregulated; FOS most significantly so. Contrary to the global GEP screening, *MIRN21* was downregulated in 3 out of 4 HMCLs. Screening results for *ATF4* and *DUSP1* could not be validated by qPCR. *Conclusions.* In 4 HMCLs we verified how $CD138^+$ cell separation upregulates FOS, a transcription factor that is upregulated in several cancers,[8] and *NFKBIA*, a regulator of the NF-kappa B pathway which is most probably of clinical importance in myeloma.[10] Neither of the 2 is part of the published high-risk myeloma GEP signatures.[2,3] MIRN21 expression levels varied according to analysis method. These results have not been clinically validated. However, we encourage that the plasma cell purification method be taken into account when comparing different myeloma risk gene signatures.

	U266	INA-6	NCI H929	RPMI 8226
FOS	*30.4 (16.0;57.5) p<0.0001*	*302 (22.1;4.13×10³) p=0.001*	*16.1 (4.24;61.0) p=0.0013*	*5.62 (2.57;12.3) p=0.0009*
NFKBIA	*2.54 (2.20;2.92) p<0.0001*	15.3 (0.858;272) p=0.0605	7.67 (0.751;78.3) p=0.0778	*1.92 (1.05;3.50) p=0.0377*
MIRN21	*0.44 (0.29;0.66) p=0.001*	*0.20 (0.10;0.37) p=0.0001*	1.12 (0.77;1.64) p=0.53	*0.42 (0.25;0.70) p=0.003*

Figure 1. Gene expression changes induced by CD138+ cell separation. Rows are genes, columns are cell lines. Cells indicate absolute fold changes (no change=1). 95% confidence intervals in parentheses. Significant (p<0.05) changes in italics.

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0341

THE ROLE OF HYPERPHOSPHORYLATED PARATARG-7 - THE FIRST AUTOSOMAL-DOMINANTLY INHERITED RISK FACTOR FOR HEMATOLOGICAL NEOPLASMS - IN MGUS, MULTIPLE MYELOMA AND LYMPHOMAS IN DIFFERENT ETHNICS GROUPS

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Background. Paratarg-7 was identified as the antigenic target of the paraproteins of 15% of IgA/IgG-MGUS and multiple myeloma (MM) and 11% of IgM-MGUS and Waldenstrom´s macroglobulinemia. We had shown that paratarg-7 was hyperphosphorylated in all patients with a paratarg-7 specific paraprotein, while pP-7 carrier state was found in only 2% of the healthy European population, thus representing a highly significant risk factor with an odds ratio of 7.9 to develop Ig/IgG-MUS and an odds ratio of 6.6 for the development for IgM-MGUS and WM (Grass et al., Lancet Oncology 2009). *Aims.* Since paratarg-7 is expressed in all tissues including peripheral blood white cells and erythrocytes, we aimed to investigate the prevalence of pP-7 in other hematological malignancies and ethnic groups. *Methods.* Lysates of peripheral blood from patients and controls were tested by gel electrophoresis and isoelectric focusing before and after phosphatase treatment. *Results.* Less Japanese IgA/IgG MGUS/MM patients had a pP-7 specific paraprotein than German patients (2/59 or 3.3%; P=0.033; χ^2-test) and the prevalence of healthy pP-7 carriers was lower (1/178) in the Japanese than in the German population (P=0.376; Fisher's exact test). However, the relative risk for pP-7 carriers to develop IgA/IgA-MGUS and MM was similar in the German and Japanese population (7.9 vs. 6.59). In order to determine whether carriers of pP-7 are at increased risk to develop hematological neoplasms other than MGUS, MM and WM, patients with DLBCL, follicular lymphoma, CLL and Hodgkin lymphoma were tested for the prevalence of pP-7. To date, no increased prevalence of pP-7 was observed in these patients. *Conclusions.* While the prevalence of the pP-7 carrier state varies between different ethnic groups, the relative risk of pP-7 to develop MGUS and MM is similar in these groups. The analysis of patients with other hematological neoplasms did not reveal an increased prevalence of pP-7 carriers among hematological diseases not associated with a paraprotein. That only MGUS/MW/MM patients who are carriers of hyperphosphorylated paratarg-7 had a paratarg-7 specific paraprotein suggests that the hyperphosphorylation of paratarg-7 induces auto-immunity and is involved in the pathogenesis of these diseases, e.g. by chronic antigenic stimulation. The identification of paratarg-7 as a frequent antigenic target enables the more detailed analysis of tumor-host interactions in these patients and its role in the pathogenesis of these diseases. Moreover, its dominant inheritance and the identification of familial cases with MGUS/MM/WM and hyperphosphorylated paratarg-7 carrier state facilitate genome-wide screens for the identification of the SNP responsible for hyperphosphorylation of this molecule.

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MULTIPLE MYELOMA CELLS ANTAGONIZE HYPOXIA INDUCED CELL DEATH THROUGH UPREGULATION BCL-2 AND BCL-XL

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Background. Hypoxia, a condition of decreased availability of oxygen, is major challenge for anticancer therapy. In solid tumors, hypoxia is considered as a double-edged sword: on the one hand, cells can be irreversibly injured and die under conditions of oxygen deficiency; on the other hand, cells that become adaptive to hypoxia are more resistant to apoptosis and less responsive to cancer therapy, resulting in relapse of the disease and transformation into more aggressive phenotypes. However, the oxygen level in bone marrow (BM) and the role of hypoxia in the pathogenesis of hematological malignancies such as multiple myeloma (MM) is still unclear. By staining the exogenous and endogenous hypoxia markers pimonidazole and HIF-1α in the bone marrow of naive and 5T33MM mice, we demonstrated that MM cells exist in a strong hypoxic niche. *Aims.* To investigate the mechanism of MM cells escaping from hypoxia induced cell death and anticancer therapy. *Materials and Methods.* The 5T33MM murine model, which mimicks the human MM disease closely, originated spontaneously in elderly C57BLKaLwRij mice. The 5T33vv MM cells were propagated by i.v. transfer of the diseased bone marrow into young recipients. The 5T33vv MM cells were isolated from 5T33MM bearing mice. Human and murine MM cell lines RPMI-8226, LP-1, 5T33vt were maintained in RPMI-1640 medium with 10% serum, supplemented glutamine (2 mM), and antibiotics (penicillin 100 U/mL and streptomycin 50 µg/mL). Hypoxic (1%, 0% O_2) conditions were established by culturing MM cells in a sealed chamber. Apoptosis was measured by flow cytometric analysis of Annexin V-APC/PI staining. Real-Time PCR and Western-blot were performed to the expression at both RNA and protein levels. Chromatin Immunoprecipitation (ChIP) was used to immunoprecipitate HIF1α-DNA complexes and to allow for the rapid PCR detection of HIF1α-regulated genes. *Results.* we found that hypoxia (1% O_2 and 0% O_2) can increase the expression of Bcl-2 and Bcl-xL at both mRNA and protein levels by qRT-PCR and Western blot. Stabilization of HIF1αenhances Bcl-2/Bcl-xL expression at both mRNA and protein levels in normoxic condition. In contrast, inhibition of the accumulation of HIF1α decreases Bcl-2/Bcl-xL expression in hypoxic condition. Furthermore, ChIP analysis provided proof that HIF1α can bind to the hypoxia response element (HRE) at Bcl-2 and Bcl-xL promoters. Antagonizing Bcl-2 and Bcl-xL with BH3 mimetic small-molecule inhibitor with high affinity to Bcl-2 and Bcl-xL, overcomes Bcl-2/Bcl-xL mediated MM cell apoptotic resistance to hypoxic stress both *in vitro* and *in vivo*, by increasing the expression of cleaved proapoptotic proteins caspase-3, 9 and PARP. BH3 mimetic small-molecule inhibitor in combination with a HIF1α inhibitor can synergistically induce MM cell apoptosis in hypoxic condition. *Conclusions.* (1) Bcl-2 and Bcl-xL are target genes of HIF1α: hypoxia-induced Bcl-2 and Bcl-xL gene expression is regulated via a HIF1α-mediated mechanism. (2) Hypoxia up-regulated Bcl-2 and Bcl-xL expression is associated with the resistance of MM cells to hypoxia induced cell death. Antagonizing Bcl-2 and Bcl-xL with BH3 mimetic small-molecule inhibitor or in combination with HIF1a inhibitor, synergistically overcomes Bcl-2/Bcl-xL mediated resistance to hypoxic stress.

0343

INHIBITION OF -TORC1 AND -TORC2 REPRESENTS A NEW APPROACH IN THE TREATMENT OF MULTIPLE MYELOMA

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Introduction: Mammalian target of rapamycin (mTOR) is a downstream serine/threonine kinase of the PI3K/Akt pathway that integrates

signals from growth factors, nutrients and stresses to regulate multiple processes, including mRNA translation, cell cycle progression and cell survival. In multiple myeloma, PI3K/Akt plays an essential role enhancing cell survival and is activated by the loss of the tumor suppressor gene PTEN and by the bone marrow microenvironment. Rapamycin analogues such as RAD001 and CCI-779 have been tested in clinical trials in MM. Their efficacy as single agents is modest, but when used in combination, they show high responses. However, total inhibition of Akt phosphorylation requires inactivation of both complexes TORC1 and TORC2. Consequently, there is a need for novel mTOR inhibitors that can target both complexes. In this study we have evaluated the activity and mechanism of action of INK128, a novel, potent, selective and orally active small molecule TORC1/2 kinase inhibitor in MM. *Methods.* The efficacy of INK128 was analyzed in eight MM cell lines (MM1S, MM1R, OPM1, OPM2, RPMI8226, U266, U266L7 and INA-6). The cytotoxicity was analyzed by means of MTT assay. Proteomic changes induced after treatment with INK128 or rapamycin were analyzed in two MM cell lines (MM1S and OPM2) with different TORC1 and TORC2 status. *Results.* We observed that INK128 fully suppressed cell viability in a dose and time dependent manner, but rapamycin reached a plateau in efficacy at±60%. The IC50 of INK128 was in the range of 7.5-30 nM in the eight cell lines tested. We checked the PI3K/Akt kinase activity and downstream signaling in all MM cell lines. MM1S showed high levels of Rictor (scaffold protein of TORC2) and p-6SR and OPM2 expressed Rictor and Raptor (scaffold protein of TORC1), alongside with high levels of p-4EBP1 and Akt kinase activity. We found that INK128 as well as rapamycin effectively suppressed phosphorylation of p6SR in both cell lines, but only INK128 was able to decrease phosphorylation of 4E-BP1, pErk and the levels of Deptor a recently described TORC1/2 interacting protein overexpressed in MM. Whereas both inhibitors had little and similar effect at 24 hour exposure, treatment of MM cells for 48 hours indicated a dose dependent increase of apoptosis much higher than rapamycin. Cleavage of PARP and Caspase-3 were found in MM cells treated with INK128 but not with rapamycin correlating with its ability to induce apoptosis. Cell cycle analysis confirmed that INK128 can cause both cell cycle arrest and death, whereas rapamycin was primarily cytostatic. Although INK128 was more potent than rapamycin to induce an effect in cell cycle progression, both drugs induced S phase decrease in all MM cell lines. INK128 displayed synergistic activity when combined with several antimyeloma agents, being most efficient in combination with dexamethasone and bortezomib. *Conclusions.* The ability of INK128 to potently induce cycle arrest and apoptosis together with its ability to augment the effect of conventional anti myeloma therapies provides the rationale for the investigation of INK128 in clinical trials for patients with MM.

0344

THE HEAT SHOCK TRANSCRIPTION FACTOR 1 IS A NEW POTENTIAL THERAPEUTIC TARGET IN MULTIPLE MYELOMA

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Background. Heat shock proteins (HSPs) like Hsp90 or Hsp70 have been found overexpressed in multiple myeloma (MM). HSPs act as molecular chaperones stabilizing a multitude of oncogenic signaling proteins and thus contribute to the malignant phenotype. Physiologically, HSPs are fast up-regulated in conditions of cellular stress. Likewise, in myeloma cells an up-regulation of HSPs was observed upon treatment with therapeutic agents, such as the proteasome inhibitor bortezomib, suggesting a potential mechanism for the development of drug resistance. However, the knowledge about regulation processes within the HSP network is limited. *Aims.* We investigated the role of heat shock transcription factor 1 (HSF1), which is supposed to act as a key regulatory factor within the HSP network, for the malignant growth of MM cells. *Methods.* Expression of HSF1 *in situ* was analyzed by immunohistochemical staining in a panel of 59 bone marrow biopsies obtained from MM patients. In addition, HSF1 and HSF1-regulated HSPs were analyzed by Western blotting in primary MM cells. To evaluate the role of HSF1 for MM cell survival, knockdown of HSF1 was performed by a pSUPER-based siRNA approach. Likewise, HSP27 knockdown was analyzed. To identify HSF1-dependent genes, expression analyses using an Affymetrix GeneChip were performed after siRNA-mediated knockdown of HSF1. Furthermore, MM cell lines as well as primary MM cells were treated with the novel pharmacologic HSF1 inhibitor triptolide either alone or in combination with the novel Hsp90 inhibitor NVP-AUY922. *Results.* Whereas HSF1 was weakly expressed in normal or MGUS plasma cells, it was strongly expressed in half of the MM biopsies *in situ* and frequently overexpressed in MM cell lines *in vitro*. HSF1 knockdown by siRNA as well as treatment with the HSF1 inhibitor triptolide led to a strong induction of apoptosis in MM cells. Importantly, also primary MM cells showed apoptosis induction after triptolide treatment. This apoptotic effect of pharmacological HSF1 inhibition was significantly enhanced by the concomitant pharmacological inhibition of HSP90 with NVP-AUY922. Inhibition of HSF1 activity caused a strong reduction of basal HSP27 and minor reduction of basal HSP70 and HSP90 protein levels. Interestingly, HSF1 inhibition blocked the induction of HSP70 and HSP27 expression after treatment with NVP-AUY922. SiRNA-mediated selective knockdown of HSP27 strongly induced apoptosis. In addition to the regulation of HSPs, gene expression analysis revealed a number of additional molecular targets of HSF1 involved in apoptosis regulation. *Conclusions.* HSF1 is frequently and strongly expressed in MM cells, protects MM cells from apoptosis and regulates the level of major HSPs like HSP90 and HSP70. Our data furthermore establish an essential role of HSF1-dependent HSP27 within the HSP network in MM. Targeting HSF1 may therefore represent an attractive potential therapeutic strategy in MM, in particular in combination with HSP90 or proteasome inhibitors.

0345

INHIBITION OF MULTIPLE MYELOMA CELL PROLIFERATION AND MAPK SIGNALING BY A DUAL PRENYLTRANSFERASE INHIBITOR

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Background. Multiple myeloma (MM) is a fatal hematologic malignancy associated with disruption of Ras-to-MAP kinase (MAPK/ERK) signaling. Ras proteins require several posttranslational modifications (e.g. prenylation, proteolysis, carboxymethylation and palmitoylation) for membrane binding and full biologic activity. Thus, use of prenylation inhibitors (e.g. farnesyltransferase inhibitors = FTIs, geranylgeranyl transferase inhibitors = GGTIs) to block Ras post-translational modification and disrupt Ras signaling is one strategy to impede oncogenic Ras function *in vivo*. Since K- and N-Ras isoforms are substrates for both farnesyltransferase (FTase) and geranylgeranyltransferase I (GGTase I), it may be necessary to inhibit both prenyltransferases in order to optimize the anti-MM response. *Aims.* In this study, we used five established MM cell lines (RPMI-8226, NCI-H929, OPM-2, L-363, and U266) to investigate the anti-MM activity of the dual prenylation inhibitor (DPI) L-778,123. *Methods.* Liquid suspension MM cell cultures were treated with serial dilutions of L-778,123 (0.08-10 μM, 96 hours) and IC_{50} values were determined by proliferation assays (MTS). *RAS* mutation status was determined by direct sequencing. Apoptosis was quantified by flow cytometric analysis of DNA strand breaks labeled using the *in situ* cell death detection kit (TUNEL assay). Levels of processed and unprocessed Ras isoforms as well as activation of MAPK-1/2 were determined by Western blotting. *Results.* Three of the five MM cell lines tested contain activating *RAS* "hot-spot"mutations (RPMI-8226: K-Ras G12A; NCI-H929: N-Ras G12D; and L363: N-Ras Q61H). Additionally, OPM-2 has been reported to contain an unusual activating H-RAS mutation. DPI L-778,123 exhibited stronger growth inhibition of multiple myeloma cells which harbour activating *RAS* mutations (e.g. NCI-H929, OPM-2, RPMI-8226, L363) versus the U266 cell line which has wild-type *RAS* (IC_{50} values were 0.54 μM, 1.97 μM, 0.73 μM and 7.86 μM vs. >10 μM, respectively). Apoptosis assays revealed no induction of DNA strand breaks upon L-778,123 treatment (0.05-5 μM; 48 and 70 hours), suggesting that the L-778,123-induced anti-proliferative effects observed in MM cells are rather cytostatic than cytotoxic. Western blotting demonstrated that FTI L-778,123 blocked prenylation of Ras family proteins. H-Ras prenylation in OPM-2 cells was inhibited after 24 hours treatment with 0.05 μM L-778,123, while N-Ras prenylation in NCI-H929 was blocked with 0.25 to 1 μM of the drug. Futhermore, L-778,123 induced an accumulation of unprocessed N-Ras proteins, perhaps reflecting an important role of prenylation in Ras protein degradation. It is also interesting to note that the IC_{50} for L-778,123 in NCI-H929 cells (0.54 μM) agrees well with the concentration of drug needed to completely inhibit oncogenic N-Ras processing in this cell line (0.25-1 μM). Western blotting experiments examining activated MAPK-1/2 levels demonstrated that L-778,123 treatment elicited a concentration-dependent inhibition of Ras down-stream signaling. *Conclusions.* These findings demonstrate that the dual prenylation inhibitor L-778,123 displays anti-MM activity. Our results support that inhibition of Ras prenylation and down-stream signaling is a major mechanism

through which L-778,123 inhibits MM cell proliferation. Alternative prenylation of K- and N-Ras by GGTase I in the presence of FTIs may explain the clinically observed incomplete response to FTI treatment. As the majority of RAS mutations in multiple myeloma occur in K- and N-RAS, treatment strategies employing dual prenylation inhibitors may exhibit more potent anti-myeloma activity than those using only farnesyltransferase inhibitors.

0346

TETRA-O-METHYL NORDIHYDROGUAIARETIC ACID (TERAMEPROCOL) INDUCES MYELOMA GROWTH ARREST AND APOPTOSIS BY INHIBITING SP1 ACTIVITY

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Background. Multiple Myeloma (MM) is characterized by uncontrolled proliferation of plasma cells associated with dysregulation of cell cycle checkpoint controls affecting mitosis. Terameprocol (TMP) is a novel clinically applicable site-specific transcription inhibitor that specifically competes with Sp1 for its DNA binding domains present on the promoter regions of number of cell cycle and growth control genes. TMP thus can regulate the function of cell cycle and apoptosis genes aberrantly expressed in many malignancies including MM. *Aims.* We investigated the *in vitro* and *in vivo* anti-MM activity of TMP alone and in combination with conventional and novel anti-MM agents. *Methods.* We examined the growth inhibitory effect of TMP against MM cells in the absence and in the presence of bone marrow stromal cells (BMSC) alone or in combination with dexamethasone and revlimid. Using the xenograft murine model of MM, we also analyzed the *in vivo* effect of TMP on MM cell growth. *Results.* TMP significantly inhibited MM cell growth in a dose-dependent fashion (IC_{50} between 5-20 μM at 24 hours) in a panel of MM cell lines as well as in primary patient MM cells. Longer exposure of MM cells to TMP resulted in reduction in survivin expression at both the gene and protein level, induction of apoptosis associated with caspase-3 and caspase-9 activation and PARP cleavage. TMP binds to GC-rich regions in chromatin and interferes with the transcription of genes that bear GC-rich motifs in their promoters. Inhibition of Sp1 activity is considered to be a major mechanism of the antitumor activity of TMP. We confirmed inhibition of Sp1 binding activity by TMP in MM cells using an ELISA assay specific for measuring Sp1 binding activity and using Sp1 sensitive luciferase reporter plasmid. Interestingly, Sp1 protein level in MM cells was not affected by treatment. We have observed induction of Sp1 activity in MM cells in the presence of bone marrow stromal cells (BMSC) and TMP was able to reverse this induction of Sp1 activity, inhibit MM cell adhesion to BMSC and overcome the positive effect of BMSC on MM cell growth. We have further confirmed the efficacy of TMP *in vivo* by treating nude mice bearing human myeloma xenograft with TMP and observing tumor regression and improved survival of mice. We finally examined if Terameprocol could enhance cytotoxicity induced by other therapeutic agents. We observed that treatment with dexamethasone and revlimid increased Sp1 activity in MM cells and treatment with a combination of these compounds with TMP had a synergistic effect on inhibition of Sp1 activity and MM cell growth compared to either agent alone. *Conclusions.* Our results confirm TMP as a specific Sp1 inhibitor and provide the rationale for clinical development of TMP alone and in combination with conventional and novel anti-myeloma agents.

0347

JAK2/STAT3 PATHWAY: A NEW TARGET OF PANOBINOSTAT IN MULTIPLE MYELOMA

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Background. Histone deacetlyase inhibitors (HDACi) represent a novel class of therapeutic agents for the treatment of solid and hematological malignancies. In multiple myeloma (MM), preclinical and clinical studies have shown that HDACi have anti-tumor activities and exhibit a synergistic cytotoxic effect when combined with conventional and novel anti-MM agents. Besides the impact on epigenetic regulation of the transcriptome, recent studies have revealed that HDACi affect the acetylation of cytoplasmic proteins/kinases mediating major signaling cascades, including JAK2/STAT3, PI3K/AKT, ERK and NF-κB pathways. These observations suggest that the antitumor effect of HDACi may include not only their impact on the histone code and chromatin remodeling, but also the function of non-histone proteins. However, the impact of HDACi on modulation of those signaling cascades in MM cells has not yet been elucidated. *Aim.* In this study, we examined the effect of Panobinostat, a class I-HDAC inhibitor currently in phase I/II clinical trial, in modulation of major signaling cascades in MM cells. Results and *Methods.* Clinically achievable doses of panobinostat were tested on MM cell lines, (MM1S, INA6 and U266). We observed that Panobinostat *in vitro* significantly inhibits the IL6-induced JAK2/STAT3 activation in MM cells at early time point by suppressed phosphorylation of JAK2/STAT3, and by reduced DNA binding activity of STAT3. These results were further confirmed under experimental conditions where the pathway was activated (ie, co-culture of MM cells with bone marrow stromal cells). We further evaluated the effect of Panobinostat on IL-6 receptor β-subunit gp130 and observed inhibition of gp130 phosphorylation at early time points, suggesting that Panobinostat inhibits IL6R/JAK2/STAT3 pathway not only by decreasing the expression of several members of the pathway, but also by modulating the function of the IL6 receptor complex as confirmed by immunoblotting and flow cytometry analysis. In contrast, Panobinostat triggers upregulation of the MAPK pathway by inducing phosphorylation of ERK ; conversely, MEK inhibitor U0126 completely blocks panobinostat-induced ERK phosphorylation, associated with synergistic growth inhibitory effect. Importantly, Panobinostat enhanced anti-MM effect induced by other conventional and novel agents and overcame protective effect by bone marrow stromal cells. Finally, we evaluated whether Panobinostat, with or without UO126, inhibits osteoclast differentiation and found that Panobinostat and U0126 trigger synergistic inhibiton of osteoclast differentiation. *Conclusions.* Our studies therefore provide new insights on mechanisms of action of HDACi not only on MM cells, but also in their microenvironment, which can inform the design of combination therapies.

0348

GOLD COMPOUND AURANOFIN HAS CLINICAL POTENTIAL AGAINST PATIENTS WITH REFRACTORY MULTIPLE MYELOMA BY TARGETING STAT3 AND NF-κB PATHWAYS

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Background. Multiple myeloma (MM) is a plasma cell malignancy that remains incurable despite the use of high-dose chemotherapy following stem cell transplantation. Novel therapeutic agents, such as thalidomide, bortezomib, and lenalidomide, have remarkable activity against both newly diagnosed and refractory patients with MM, but prolonged exposure may result in the development of *de novo* drug resistance. It is necessary to identify and validate novel agents with less toxicity to overcome drug resistance and to improve clinical outcome. Auranofin (AF) is a coordinated gold compound that has been widely used for the treatment of rheumatoid arthritis based on its anti-inflammatory properties through the inhibition of NF-κB activation. *Aims*. Assuming that AF has the potency to induce apoptosis in MM cells by interfering with NF-κB pathways, it may be a candidate for novel therapeutic agents. *Methods.* We here examined the efficacy and molecular mechanisms of action of AF for various MM cell lines and fresh samples from patients with MM *in vitro*. Furthermore, the clinical benefits of daily oral administration of AF (6 mg per day) in 7 patients with refractory MM with a signed informed consent were examined. *Results*. AF inhibited the growth of MM cells in a time- and dose-dependent manner with IC_{50} of 50 nM at 24 h. AF significantly induced cell cycle arrest at the G1 phase and subsequent apoptosis of MM cells, involving the activation of caspases-3, -8, and -9. AF also induced apoptosis in CD138-positive plasma cells from patients with MM. Treatment with AF inhibited the constitutive and IL-6-induced activation of STAT3, and then down-regulated the expression of Mcl-1 but not that of Bcl-2 or Bcl-xL proteins. To clarify the biological significance, Mcl-1 expression vector (pEGFP-hmcl-1) and control vector were introduced into U266 cells (designated as U266/mcl and U266/neo cells). Induction of apoptosis by AF was abrogated in U266/mcl, but not in U266/neo cells. Further, electrophoretic mobility gel shift assay demonstrated that IL-6-induced STAT3 binding activity was inhibited by the presence of AF. In addition, AF down-regulated the activation of NF-κB in an IκB-independent manner, and also inhibited DNA binding activity. Finally, since 2008, after the approval of the pro-

tocol by Ethic Committee of the Saitama Medical University, 7 patients with refractory MM were treated with oral AF 6 mg daily. The median follow-up for patients was 9 months. All 7 patients responded to AF, including one partial response and 6 stable diseases. No adverse events have been observed to date. Details of the clinical outcome will be presented. *Conclusions*. Gold compound AF inhibited constitutive activation of both STAT3 and NF-κB, resulting in the down-regulation of Mcl-1 in MM cells with clinical relevance. A low pharmacological concentration (50 nM) of AF is widely employed for the treatment of rheumatoid arthritis without any side effects; therefore, it may be used to treat MM without severe toxicity. We propose that AF may have potency as a new molecular-targeted agent for the treatment of MM.

0349

PRE-CLINICAL APPROACH OF MULTIPLE SIGNAL TRANSDUCTION PATHWAYS INHIBITION IN MULTIPLE MYELOMA

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Background. Multiple myeloma (MM) is a plasma cell malignancy incurable with existing conventional therapies. Advances in the understanding of the underlying molecular mechanisms of growth, progression and drug resistance of neoplastic cells has allowed the development of promising new agents that inhibit specific proteins aberrantly regulated in key signal transduction pathways. These agents have shown promising results in pre-clinical trials and some of them are currently at the early stages of clinical investigation. However, since it has become progressively more evident that inhibition of a single kinase may frequently induces escaping mechanisms in neoplastic cells trough activation of cross-talking pathways, there is a clear need to identify agents effective on inhibition of multiple targets. We already showed in *in vitro* studies on MM cell lines and primary CD138$^+$ cells from MM patients the potent growth-inhibitory effects of the specific MEK inhibitor PD0325901 and the markedly pro-apoptotic activity of the BH3-mimetic ABT-737 (kindly provided by Abbott Laboratories). In addition, the activity of Mevinolin, a mevalonate pathway inhibitor, on inducing apoptosis of MM cells by regulating different signal, including the MEK/ERK module, has been reported. *Aim.* To analyze the effects of the simultaneous inhibition of MEK/ERK, Bcl2/BclXL and mevalonate pathways on apoptosis and cell growth of MM cell lines and primary samples. *Results.* MM cell lines (KMS18, KMS27, ARH-77) were exposed to increasing concentrations of PD0325901 (1-100 nM), ABT-737 (1-100 nM) and Mevinolin (1-100 μM), alone and in combination. When used as single agents the inhibition of cell-growth was dose-dependent, while if used in combination the inhibition of cell growth was synergistically enhanced, with a combination index (CI) of 0.12 and 0.15 for the PD0325901+ABT-737 (PD-ABT) and PD0325901+Mevinolin (PD-MEV) combination, respectively. We then investigated the effects of these agents on apoptosis, demonstrating that PD0325901 as single drug mainly showed cytostatic effects without affecting apoptosis, while both ABT-737 and Mevinolin induced apoptosis only at high concentrations. The simultaneous exposure to PD0325901 with ABT-737 or Mevinolin was able to induce apoptosis at lower concentrations, showing that these pro-apoptotic interactions are highly synergistic in nature, with a CI of 0.2 (KMS18) and 0.17 (KMS27) for PD-ABT and of 0.135 (KMS18) and 0.128 (KMS27) for PD-MEV. Similarly, mitochondrial membrane depolarization was greatly induced by both combinations. SUMMARY: In conclusion, we demonstrated that the simultaneous disruption of MEK/ERK and Bcl2/BclXL or mevalonate signalling results in higher apoptosis induction and growth inhibition of MM cells, supporting the adoption of this approach based on the simultaneous multiple signal transduction pathway inhibition.

0350

BORTEZOMIB DOWN REGULATES TELOMERASE ACTIVITY IN MULTIPLE MYELOMA - DIFFERENT LEVELS OF REGULATION IN CELLS FROM VARIOUS SOURCES - IN VITRO AND EX VIVO STUDIES

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Background. The proteasome inhibitor bortezomib is considered an effective drug in Multiple Myeloma (MM). However, its exact mechanism of action is not yet fully understood. The importance of telomerase in the biology of MM and as a prognostic factor has been clinically established, but has not been assessed in response to bortezomib. In light of common signaling pathways of bortezomib function and telomerase regulation we sought to explore the possible effect and mechanism of bortezomib on telomerase activity in MM cells. *Aims.* To characterize the effect of bortezomib on telomerase activity (TA) and its regulation in MM cell lines and in ex vivo MM cells. *Methods.* Two MM cell lines, ARP-1 and CAG and cells purified from patients' marrow were exposed to bortezomib. TA was determined by the TRAP assay. Its regulation was evaluated on the epigenetic level by assessment of the methylation status of the hTERT promoter (by bisulfite conversion assay) and on the transcriptional level by measuring the expression of the hTERT gene (by real time PCR). The binding ability of three hTERT transcription factors; SP1, c-Myc and NFκB was evaluated by the ChIP assay. Posttranslational phosphorylation of the enzyme was determined by following the phosphorylation levels of Akt and PKCα (by Western blotting). Cell proliferation was assessed by the WST-1 viability reagent. *Results.* Bortezomib inhibited telomerase activity *in vitro* in both cell lines and ex vivo cells, but in a different kinetic manner. In all cells the transcription of hTERT was inhibited by the drug. This inhibition was mediated by a decreased binding of SP-1 to its promoter while NFκB and c-Myc binding were unaffected. The methylation status of a portion of the hTERT promoter was not changed. On the posttranslational level, the phosphorylation of PKCα, the main kinase of telomerase, was decreased only in the ARP-1 cell line explaining the delayed onset of telomerase inhibition in CAG cells. *Conclusions.* This study establishes telomerase as a novel target of bortezomib in MM. The differential mechanism of inhibition (both on the level of regulation and in kinetics) in cells from different sources may indicate that similar differences between patients with MM may translate to the clinical setting as well. More studies are needed to determine whether telomerase activity in response to bortezomib may serve as prognostic factor in patients with MM.

0351

THE HDAC INHIBITOR LBH589 ENHANCES THE ANTI-MYELOMA EFFECT OF THE IGF-1 RTK INHIBITOR PICROPODOPHYLLIN

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Our previous studies have shown that inhibition of IGF-1R pathway by picropodophyllin (PPP) can be achieved with favorable therapeutic window in multiple myeloma (MM) models *in vivo* and *in vitro*. Considering the acquirement of compensatory genetic lesions during MM progression approaches targeting single receptors are however not likely to be curative for MM. In line with this notion remaining MM cells in the *in vivo* 5TMM model eventually leads to relapse and mortality. To counteract this, we combined PPP in a combinatorial drug screen (HTS). In this approach we focused on the use of one of these candidates, the HDAC inhibitor LBH589, in combination with PPP in MM models *in vivo* and *in vitro*. The studies show that PPP and LBH589 used at suboptimal concentrations has synergistic effects in a combinatorial regimen both *in vitro* and *in vivo*. The contribution from the single drugs and the combination were monitored for apoptosis, cell cycle distribution, and the impact on downstream gene and protein expression in human and mouse MM models *in vitro*. In the RPMI8226 human MM cell line, simultaneous treatment with both compounds for 48h caused a 5-fold increase of apoptotic and late apoptotic/necrotic cells as compared to controls, while treatment with either compound alone only induced a 3-fold increase. After 24h cleavage of apoptotic proteins caspase -9, -8 and -3 could be found in RPMI8226 cells treated with both drugs individually, but in the combination we observed an additive effect on the cleavage of the active forms of caspase 8 as compared to single drug treatments. The combination of LBH589 and PPP could be monitored as an accumulation of cells in the G2/M phase, and subsequent down-regulation of cell cycle regulated proteins. The effect of both compounds on the expression of cyclin B1, -E and -D2 was additive, as demonstrated by western blot. Similar *in vitro* experiments using cells of the 5T33MM murine model confirm these data. At lower combinatorial concentrations, a significant additive reduction of VEGF secretion (56%) when compared to the single treatments (45% and 13% for LBH589 and PPP respectively) of 5T33MM cells was observed. Finally, we examined the *in vivo* effect of the combinatorial treatment using sub-optimal con-

centrations of LBH589 (2.5 mg/kg/day i.p. injection) and PPP (1,5mg/day orally) and assessed the overall survive rate using Kaplan-Meier analysis. Combined treatment resulted in a significant (P<0.03) prolonged survival (47.5 days) when compared to the control group (22 days), the LBH589-treated group (32.5 days) and the PPP-treated group (25 days). In conclusion, the results indicate an improved MM treatment opportunity in using a combination of PPP and LBH589.

0352

ACTIVATION OF INNATE IMMUNITY IN PATIENTS RECEIVING COMBINATION THERAPY WITH LENALIDOMIDE (REVLIMID), CYCLOPHOSPHAMIDE AND LOW-DOSE DEXAMETHASONE (RCD) FOR NEWLY DIAGNOSED OR RELAPSED/REFRACTORY MYELOMA

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Background. Lenalidomide, a derivative of thalidomide, has been reported to exert immunomodulatory activity by stimulating T cell and NK cell mediated immune response. However, the exact mode of activation is as yet unknown. The purpose of our study is to assess the effect of a combination chemotherapy including lenalidomide on NK/NKT cell numbers and expression of activating receptors (NKG2D, NKp30, NKp44 and NKp46) in patients with newly diagnosed or relapsed/refractory multiple myeloma (MM). *Patients and Methods.* Patients received lenalidomide (25 mg d1-21), dexamethasone 40 mg (d1, d8, d15, d22) and cyclophosphamide (500 mg as iv infusion d1 in cycle 1, d1 and d8 from second cycle onward). The next cycle was started on d29. Immediately before start of a cycle blood was drawn for flowcytometrical analysis of lymphocytes. *Results.* Currently, samples of 10 patients with multiple myeloma (median age 71 years, interquartile range (IQR) 68-73 years) have been analysed. In all patients RCD was administered at full dose. Five patients received RCD as first line therapy and five had relapsed/refractory MM. After a median of 2 cycles 90% of the patients achieved at least a partial remission (PR) (one minimal response, eight PR, one very good PR). In contrast to other anti-myeloma regimens the side effect profile of this combination was highly predictable, only one case of grade III neutropenia was noted. When comparing the data before start of therapy with data before the second cycle, the absolute number of lymphocytes decreased from median 1570/µL (IQR 1185-1590) to 1270/µL (IQR 1150-1825). In contrast, NK cells and NKT cells increased from 121.9/µL (IQR 117-253) to 210.9/µL (IQR 152-294, NK cells) and 88.8/µL (IQR 60-216) to 128.4/µL (IQR 32-319, NKT cells). In addition, the expression of activating receptors increased. The percentage of $NKG2D^+$ NK cells increased from 91.5% (IQR 88.6-93.5) to 93.9% (IQR 92.1-94.3) and $NKp30^+$ NK cells increased from 22.3% (IQR 16.7-40.6) to 26.6% (IQR 20.2-51.6). Also, NK cells expressing NKp44 increased significantly (median 0.115% (IQR 0.01-0.58) to 0.64% (IQR 0.3-0.97), P=0.03). In contrast, no difference was noted in NKp46 expression. Comparing NKT cells, the expression of $NKG2D^+$ increased from 74.9% (IQR 69.4-88.5) to 82.6% (IQR 75-84.2). The expression of the other markers of NKT cells remained unchanged. Updated results of a larger group of patients will be presented. *Conclusions.* These findings provide clinical data for the immunomodulatory activity of lenalidomide, which leads to an increase and activation of NK and NKT cells very early in the course of treatment and despite the concomitant application of immunosuppressive drugs.

0353

SYNERGISTIC CYTOTOXIC EFFECT OF COMBINED TREATMENT WITH NANOSIZED REALGAR PARTICLES AND SULFORAPHANE IN MULTIPLE MYELOMA CELLS IN VITRO

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Background. In Western pharmacological history approximately 60 different arsenic preparations have been developed for medicinal use. Traditional Chinese medicines employ different form of mineral arsenicals (orpiment - As2S3, realgar - As4S4, and arsenolite - As2O3, arsenic trioxide) and realgar (REA) alone is included in 22 oral remedies based on Chinese Pharmacopeia Committee. Clinical use of arsenic trioxide (ATO) may be limited due to its acute toxicity as well as its association with carcinogenesis. REA is less toxic than ATO but exerts potent antileukemic activity in combination with imatinib. *Aims.* Recently we have disclosed cytotoxicity potentiation of another metal - cisplatin with synthetic isothiocyanate E4IB. Therefore the aim of present study was to characterize the cytotoxicity of REA as well as in combination with sulforaphane. *Methods.* The multiple myeloma cell lines OPM1, RPMI-LR5, and U266 were treated with different suspensions of REA particles with diameters ranging from 100 to 230 nm prepared by high-energy milling. The cell growth was tested by the MTT assay after 72 h of treatment. As some preparations of nanosized REA exerted high absorbance in MTT assay the direct cell counting with reference beads or CFSE-stained cells (x) using flow cytometry was performed. Viability (fluorescein diacetate/propidium iodide), apoptosis (Annexin-V binding) and mitochondrial potential (JC-1) were determined by standard flow cytometric assays. The CalcuSyn software was used to evaluate whether the combined sulforaphane and REA treatment was additive, synergistic or antagonistic. *Results.* Nanosized REA induced time- and concentration-dependent cell cytotoxicity with IC_{50} within the range of 0.1-0.3 microgram/mL of As. Apoptotic (Annexin-V+ PI-) and late apoptotic (Annexin-V+PI+) cells were observed as well as a decrease of mitochondrial potential. Combined treatment of cells with REA and sulforaphane resulted in enhanced cytotoxicity that was evaluated as synergistic one. *Summary*. Combined use of REA nanoparticles and sulforaphane might provide a promising therapeutic approach for multiple myeloma treatment especially in the context of low systemic damage of REA in comparison to ATO. *The support through the Agency for Science and Development (Project VVCE-0001-07), the Slovak Grant Agency (VEGA 2/0119/08 and 2/7059/27) is gratefully acknowledged.*

0354

INHIBITION OF MAGEC1/CT7 SIGNIFICANTLY DECREASED THE NUMBER OF SKO-007 CELLS IN G2 PHASE OF THE CELL CYCLE AND INCREASES THEIR SENSITIVITY TO BORTEZOMIB.

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Background. MAGEC1/CT7 gene encodes for a cancer testis (CT) antigen frequently expressed in multiple myeloma (MM) that may be a potential target for immunotherapy in this still incurable disease. This CT gene expression is restricted to malignant plasma cells and it has been demonstrated a positive correlation between *MAGEC1/CT7* expression and advanced stage of MM. The exact function of this protein for tumor biology is not yet understood, but same findings suggested that *MAGEC1/CT7* play pathogenic role in MM, possibly in the dysregulation of the cell cycle. *Aims.* The aims of this study were to elucidate the role of *MAGEC1/CT7* in the control of cellular proliferation, invasive potential and regulation of cell cycle of Sko-007 myeloma cell line and to evaluate the impact of this gene silencing in cells treated with the proteasome inhibitor bortezomib. *Methods.* Short hairpin RNA (shRNA) specific for *MAGEC1/CT7* was previously inserted in the pRS (pRETROSUPER) retroviral vector. The pRS-shRNA-*MAGEC1/CT7* was co-transfected with pCL-amphotropic packing vector in 293T cells to produce virus particles. Sko-007 myeloma cell line was transduced for stable expression of shRNA-MAGEC1/CT7. Downregulation of *MAGEC1/CT7* was confirmed by Western Blot and Real Time PCR (RQ-PCR). Functional studies included cell proliferation, 3H thymidine incorporation, invasion potential using Matrigel-coated Transwell filters and cell cycle analysis with bortezomib or without using propidium iodide (PI). *Results.* Sko-007 was chosen to perform the functional studies because basal *MAGEC1/CT7* gene expression level was higher in this cell line when compared with other myeloma cell lines (U266 and SK-MM-2). Sko-007shMAGEC1/CT7Δ (scramble, antisense strand deleted - GC bases) was used as control for all the experiments. *MAGEC1/CT7* expression was ~5 times lower in Sko-007sh*MAGEC1/CT7* than control cells by RQ-PCR. Western Blot showed ~80% decrease in *MAGEC1/CT7* protein expression by Sko-007shMAGE1/CT7 when compared with Sko-007shMAGEC1/CT7Δ . Our functional assays did not show any difference in cell proliferation and DNA synthesis when Sko-007sh*MAGEC1/CT7* was compared with control cells. Sko-007 cell line showed invasive potential, but no difference was observed in Sko-007sh*MAGEC1/CT7* or control cells.

Using flow cytometry analyses, we detected alterations between Sko-007sh*MAGEC1/CT7* cells and the control cells in G1 (P=0.0257), S (P=0.0058) and G2 (P=0.0088) phases. Sko-007sh*MAGEC1/CT7* population has 40% decreased in G2 phase when compared to control cells. When both inhibited and control cells were treated with 10 nM bortezomib, Sko-007sh*MAGEC1/CT7* population presented in G2 phase was more sensitive to drug (61%) than control cells (16%) (P=0.0475). *Summary/Conclusions.* Expression of *MAGEC1/CT7* mRNA could be inhibited by the shRNA strategy, although the concomitant reduction of its protein level did not seem to have an impact in cell proliferation, DNA synthesis or in invasive potential of Sko-007 MM cell line. However, the inhibition of *MAGEC1/CT7* significantly decreased the number of Sko-007sh*MAGEC1/CT7* cells in G2 phase of the cell cycle *in vitro* and these cells also appear more sensitive to bortezomib than control cells (Supported CNPq and LICR São Paulo Branch).

0355

PROTEIN PHOSPHATASE 2A AS A PROGNOSTIC MARKER OF MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE (MGUS) AND MULTIPLE MYELOMA (MM)

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Background. Proteinphosphatase 2A (PP2A) is a heterotrimeric holoenzyme, the activity of which is regulated by phosphorylation of tyrosine 307 of the catalytic C subunit. Since 2 bands with different signal intensities appear after isoelectric focusing (IEF), of which the upper band represents a variant of PP2A with a phosphorylation of tyrosine 307, we determined whether a correlation exists between the different PP2A states in healthy donors and MGUS/MM patients. *Methods.* Blinded samples of 169 consecutive patients and healthy donors were analyzed. Since the total amount of PP2A was similar in patients and controls, the ratio between the phosphorylated and the non-phosphorylated form could be determined by densitometry. In addition, the activity of PP2A was determined by immunoprecipitation and measurement of the phosphate turnover. *Results.* Samples of 30 consecutive MGUS and 73 MM patients as well as of 66 healthy donors were sorted by ascending ratio and plotted versus the diagnosis. MM patients had a higher ratio due to the more intense upper signal and less activity as shown by a shift to the phosphorylated variant compared to healthy donors, while in healthy controls a low ratio was associated with high activity. Results in MGUS patients were intermediate between MM patients and healthy donors. The 3 cohorts of healthy controls, MGUS and MM patients differed significantly with respect to their PP2A phosphorylation (P<0.001). There was no difference with respect to age and sex. Similarly, when selected clinical parameters, such as IgG, IgA, IgM, β2-microglobulin, total protein, albumin and creatinine of 12 MGUS/MM patients each, and 13 healthy controls were also plotted versus the ratio, no correlation with PP2A phosphorylation was found. Same was seen by analysis of patients with samples taken within 4 weeks after chemotherapy. *Conclusions.* Since the differences between healthy donors and MGUS/MM patients are significant in terms of the ratio between the two PP2A phosphorylation forms and activity of PP2A, determination of the two phosphorylation forms and PP2A activity might be helpful in the follow up of patients with MGUS who are at high risk to develop MM.

0356

LENALIDOMIDE TREATMENT IS ABLE TO RESTORE REGULATORY T CELLS IN MULTIPLE MYELOMA PATIENTS

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Background. Multiple myeloma (MM) is a malignant plasma-cell proliferative disorder associated with dysfunctional T-cell responses. Lenalidomide appears to be a promising agent for the treatment of myeloma but its exact antitumor mechanism of action is unknown although an immunomodulatory effect has been postulated to be responsible for at least some of its activity. We therefore evaluated two lymphocytes subsets: the T-regs ($CD4^+/CD25^+/FOXP3^+$) and the T lymphocytes $CD3^+$ bearing $CD200^+$ (a tolerogenic molecule) in MM patients at diagnosis and we followed these population during treatment with lenalidomide and dexamethasone. *Materials and Methods.* Fifteen patients with MM (median age 54 years) received, as first line therapy, 4 cycles of lenalidomide 25 mg daily on days 1 to 21 of a 28-day cycle and dexamethasone 40 mg daily on days 1, 8, 15, 22 of each cycle. All patients were evaluable for response and toxicity. Peripheral blood mononuclear cells (PBMNc) were obtained from MM patients using density gradient centrifugation (Fycoll), at the beginning of each cycle and at the end of therapy. The absolute number of T-reg and of CD200 positive T- lymphocytes, were evaluated by cytometry. Thirty healthy subjects were used as control. *Results.* MM patients at diagnosis have a significantly lower rate of T-reg and T- lymphocytes bearing CD200+ than normal (47,2±35,5/mmc and 40,3±27,7 /mmc vs 64,2±20,9 and 75,4±47,3)(P=0,006 and P=0,02 respectively). Lenalidomide + dexa treatment resulted in an increase of both Treg cells and T-lymphocytes $/CD200^+$ already after the first cycle of treatment with a small decrease at the end of treatment. (Table 1). *Conclusions.* Our data emphasize the role of lenalidomide in modulating the endogenous tumor-specific immune response and underline the anti-myeloma activity of these new class of drugs.

Table 1.

PTS (n) 15	CD3+CD200+ /mmc	CD4+CD25+FOXP3+/mmc
Cycle	Means± SD	Means± SD
Healthy controls	75,4±47,3	64,2±20,9
Pre-therapy	40,3±27,7	47,2±35,5
Post-I Cycle	90±50*	68±31*
Post-II Cycle	83±76	74,4±40
Post-III Cycle	97,4±71*	62±36*
Post-IV Cycle	62,3±28	52,3±33
*= P < .05 by test Mann Whitney. The correlation is made with Pre-therapy.		

Myeloma and other monoclonal gammopathies - Clinical 1

0357

WITHDRAWN BY AUTHOR

0358

PHASE 3B UPFRONT STUDY: INTERIM RESULTS FROM A COMMUNITY-BASED PROSPECTIVE RANDOMIZED TRIAL EVALUATING THREE BORTEZOMIB-BASED REGIMENS IN ELDERLY, NEWLY DIAGNOSED MULTIPLE MYELOMA PATIENTS

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Background. This US community-based phase 3b study compared the safety and efficacy of three bortezomib (Vc)-based regimens for multiple myeloma (MM), Vc-dexamethasone (VcD), Vc-thalidomide-dexamethasone (VcTD), and Vc-melphalan-prednisone (VcMP), in previously untreated MM patients ineligible for high-dose therapy and transplantation. A preplanned interim analysis was performed after 70 patients in each arm had had the opportunity to complete 4 cycles of therapy, with the intention of stopping enrollment in the inferior arm. An Independent Data Monitoring Committee (IDMC) reviewed all efficacy, safety, and quality-of-life data. There was no difference (≥10%) in very good partial response or better (≥VGPR) rates between any two treatment arms, and safety data were similar. The IDMC recommended continuing enrollment in all three arms, and performing a second interim analysis after longer treatment duration, the results of which are presented here. *Methods.* Patients were randomized (1:1:1) to receive 49 weeks of therapy: 24 weeks (eight 21-day cycles) of induction with VcD, VcTD, or VcMP (Vc 1.3 mg/m^2, days 1, 4, 8, 11; D 20 mg, days 1, 2, 4, 5, 8, 9, 11, 12 [Cycles 1-4], days 1, 2, 4, 5 [Cycles 5-8]; T 100 mg/d, days 1-21; M 9 mg/m^2 and P 60 mg/m^2, days 1-4, every other cycle), followed by 25 weeks (five 35-day cycles) of maintenance with Vc alone (1.6 mg/m^2, days 1, 8, 15, 22). The primary endpoint was progression-free survival. The second interim analysis was performed after 100 patients in each arm had had the opportunity to complete 8 cycles. *Results.* Patients in the VcD, VcTD, and VcMP arms had median ages of 73.5, 73, and 72 years; 85%, 63%, and 73% had ISS stage II/III MM; and 21%, 27%, and 27%, respectively, were non-Caucasian. The overall response rate (best confirmed response) was 69%, 79%, and 72% in the VcD, VcTD, and VcMP arms, respectively (including 36%, 44% and 39% ≥VGPR). In the VcD, VcTD, and VcMP arms, respectively, 69%, 82%, and 79% of patients experienced grade ≥3 adverse events (AEs), 48%, 55%, and 46% experienced serious AEs, and 26%, 42%, and 31% discontinued due to AEs. The rate of peripheral neuropathy (PN) was highest in the VcTD arm (59%, versus 41% [VcD] and 43% [VcMP]); similar trends were seen for the rate of grade ≥3 PN (25%, versus 14% and 19%, respectively). Rates of DVT were 7%, 4%, and 2% with VcD, VcTD, and VcMP, respectively. The most common AEs resulting in discontinuation (≥3 patients overall) in the VcD, VcTD, and VcMP arms were PN (8%, 18%, 17%), weakness (1%, 2%, 3%), fatigue (2%, 0, 1%), congestive heart failure (1%, 2%, 0), and pneumonia (0, 2%, 1%). *Conclusions.* All three regimens were active with well-characterized and predictable toxicities in this large, phase 3b study, which was primarily conducted in community-based oncology centers and included a high proportion of non-Caucasian patients. After both interim analyses, the efficacy and safety of the three regimens were deemed similar by the IDMC. Patients continue to be followed for assessment of progression-free survival.

0359

PROGNOSTIC IMPACT OF QUALITY OF RESPONSE IN PATIENTS WITH MULTIPLE MYELOMA RECEIVING UP-FRONT THALIDOMIDE-DEXAMETHASONE AND DOUBLE AUTOLOGOUS STEM-CELL TRANSPLANTATION (ASCT)

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Aim of the present study was to evaluate the prognostic impact of high-quality response (e.g. VGPR vs CR) in a large series of younger patients with symptomatic multiple myeloma (MM) who were planned to receive up-front thal-dex and double ASCT to support high-dose melphalan (200 mg/m^2). By study design, thal (200 mg/day) and pulsed low-dose dex (160 mg/month), were administered from the outset until the second ASCT. The analysis was performed on an intention-to-treat basis on a total of 357 patients who were followed for a median of 43 months. More than 80% of the patients were screened at diagnosis for the presence of cytogenetic abnormalities by FISH analysis. Forty-five percent of patients had del(13q), while t(4;14) and del(17p) were found in 14% and 6% of patients, respectively. The rate of at least VGPR increased from 31% after thal-dex induction therapy to 60% after the second ASCT. The final rate of immunofixation negative CR was 33% for all the patients and 44% for those who actually received double ASCT. The median duration of CR was 66 months. Median TTP and PFS were 68 and 47 months, respectively, with 5-year projected rates of 45% and 33%. The 5-year projected OS rate was 65%. Median OS after relapse or progression was 30 months, suggesting that short-term thal exposure had no adverse influence on response to subsequent salvage therapies. The quality of best response influenced clinical outcomes. In particular, patients achieving CR had significantly longer PFS and OS than patients in VGPR (PFS: median 68 vs 48 months, respectively, P=0.04; 5-year projected OS 84% vs 72%, respectively, P=0.02). Similarly, patients in VGPR had better outcomes compared with patients achieving PR (P=0.02 and P=0.04 for PFS and OS comparisons, respectively). The impact of high quality responses on PFS and OS was statistically significant also in patients with poor-risk cytogenetics, as identified by the presence of t(4;14) and/or del(17p). In particular, median PFS was 46 months for patients with at least VGPR vs 16 months for those in PR, P=0.0000. The corresponding OS values (projected at 5 years) were 75% vs 0%, P=0.0000. Patients achieving at least VGPR after thal-dex induction therapy had a significantly longer PFS in comparison with those who failed this objective (5-year projected rate: 58% vs 44%, respectively , P=0.04). In a multivariate analysis, attainment of CR and low serum beta2-m levels were the most important variables significantly extending TTP (P=0.04), PFS (P=0.000) and OS (P=0.003). In conclusion, attainment of CR and VGPR was a major determinant of favorable outcomes for patients treated with thal-dex incorporated into double ASCT. The prognostic value of the quality of response remained statistically significant even in patients with high-risk cytogenetics. Attainment of high quality response after induction therapy predicted for improved PFS.

0360

TREATMENT WITH LENALIDOMIDE OVERCOMES THE POOR PREDICTIVE INFLUENCE OF HIGH IRF4 IN PATIENTS WITH MULTIPLE MYELOMA

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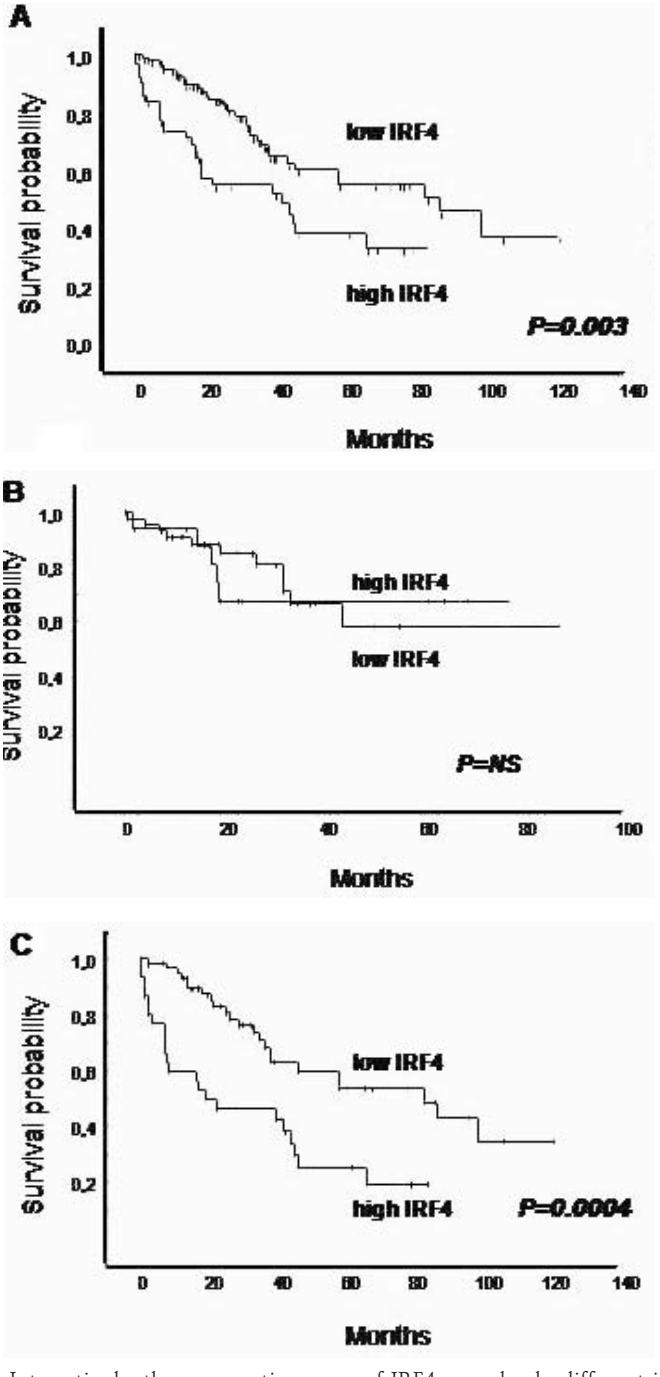
Background. Interferon regulatory factor 4 (IRF4) is essential in B cell differentiation, functions as positive regulator of transcription, and depletion of IRF4 results in rapid apoptosis of myeloma cells. Previously, we have reported that high IRF4 expression in myeloma cells is associated with shortened survival. Lenalidomide exposure of myeloma cells results in significant inhibition of IRF4 and in enhanced expression of suppressor genes. However, the relationship of IRF4 expression in myeloma patient samples and clinical outcome after lenalidomide ther-

apy has not been explored until now. *Aims and Methods.* We measured IRF4 mRNA expression in bone marrow samples of 154 well characterized multiple myeloma patients (median age: 65 years, range 33-90 years) using real time PCR, and evaluated the predictive value of IRF4 in association with lenalidomide and other treatment strategies. *Results.* The median IRF4 expression of patient samples was 4.6 fold relative to normal PBMNC (range: 0.1-15343.4). Overall survival analysis confirmed previous data, showing a significantly shorter overall survival for patients with high IRF4 expression (defined as >13 fold as compared to normal PBMC), with a medium survival of 42 months in high vs. 83 months in low expressers (P=0.003, Figure 1A).

Figure 1.



Interestingly, the prognostic power of IRF4 was clearly different in patient subgroups. Sixty-four patients were treated with lenalidomide while 90 patients received other therapies including high dose therapy, bortezomib, thalidomide, alkylating agents and corticosteroids after bone marrow collection. In patients with lenalidomide therapy, there was no significant difference regarding overall survival between patients with low or high IRF4 anymore (Figure 1B). In contrast, in patients treated otherwise, high IRF4 was not only again associated with a shorter overall survival, the prognostic significance was even pronounced, with a median survival of 19 vs. 83 months (P=0.0004, Figure 1C). When patients were stratified into 4 groups according to IRF4 expression and treatment with or without lenalidomide, those with high IRF4 without lenalidomide therapy had a significantly worse prognosis, while in the other 3 groups overall survival curves were similar. In high IRF4 expressers, patients treated with lenalidomide had a significant better overall survival than those receiving other therapies (median: not reached vs. 19 months, P=0.02). In low IRF4 expressers, no difference regarding overall survival was found between patients treated with or without lenalidomide. To date, information on clinical response to lenalidomide therapy is available in 38 patients. One patient had a complete remission (CR), 3 had a near complete remission (nCR), 10 had a partial response (PR), 5 had a minimal response (MR), 6 had stable disease (SD), and 13 had progressive disease (PD). Of note, 3 of 4 patients with a CR or nCR were high IRF4 expressers, while 10 of 13 patients with PD were found in the low IRF4 group. *Summary/Conclusions.* In our study, the negative prognostic impact of high IRF4 was overcome by treatment with lenalidomide. IRF4 expression might be used as a predictive factor to optimise treatment, especially for the 30% of myeloma patients with high IRF4 expression.

0361

ZOLEDRONIC ACID THERAPY VERSUS CONTROL IN PATIENTS WITH MULTIPLE MYELOMA IN STAGE I (DURIE & SALMON): RESULTS OF A PHASE III STUDY OF THE DSMM AND OSHO

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Background. Bone disease is a hallmark of multiple myeloma. Occurring in the majority of myeloma patients, it is associated with bone pain, fractures, hypercalcemia and has major impacts on quality of life. In a cohort study, 16 times more fractures were observed than expected in the year before diagnosis. Before lytic lesions become apparent, a high rate of bone resorption is already existent in the majority of the patients. The standard of care in asymptomatic multiple myeloma is "wait and see". Due to their inhibitory effect on bone resorption, bisphosphonates might prevent skeletal-related events in early myeloma. Furthermore, bisphosphonates might be beneficial by interrupting the vicious cycle between osteoclast activation and myeloma progression or by other antitumor effects. *Aims.* Zoledronic acid is a third generation bisphosphonate which induces a strong inhibition of bone resorption. The present phase III study investigated the effect of intravenous treatment with zoledronic acid on progression-free survival (PFS) in patients with stage I multiple myeloma. The primary objective of the trial was PFS, secondary objectives comprised time to develop skeletal-related events and overall survival, as well as tolerability and safety of zoledronic acid. *Methods.* This prospective, randomized, open multicenter study started in 2000. PFS is calculated from start of the treatment to disease progression or death. Progression was defined as progression in stage II or III (Durie & Salmon), progression of osteolytic lesions or occurrence of skeletal-related events. Ethical approval and informed consent from all patients were obtained. It was planned to include 220 patients, but due to slow recruitment the study was prematurely terminated. 140 patients were available for analysis (71 zoledronic acid and 69 control). Patients aged > 18 years with a diagnosis of multiple myeloma according to the criteria of the British Columbia Cancer Agency (which are similar to IMWG criteria published later) and Stage I (Durie and Salmon) were enrolled in this clinical trial. Zoledronic acid (4 mg i.v.) was administered every 4 weeks. Treatment was planned to last until progression or 48 months, whatever comes first. *Results.* For the incidence of skeletal-related events, a trend favoring treatment with zoledronic acid was detected (P=0.056). Of interest, no patient in the zoledronic acid group experienced skeletal related events. Progression according to the definition of the protocol occurred in 19 patients treated with zoledronic acid and 26 patients in the control group, respectively (26.8% vs. 37.7%). Kaplan-Meier plots for progression-free survival

showed a trend favoring zoledronic acid, but the difference did not reach statistical significance (log-rank: P=0.34). *Conclusion*s. This phase III study comparing zoledronic acid with no treatment in multiple myeloma patients in stage I according to Durie & Salmon showed a trend favoring zoledronic acid in regard of both skeletal-related events and progression of multiple myeloma, although a statistical significance was not reached, possibly due to the fact that the planned sample size was not recruited.

0362

INTERNATIONAL STAGING SYSTEM (ISS) IS A GOOD PROGNOSTIC FACTOR FOR PROGRESSION FREE SURVIVAL IN MULTIPLE MYELOMA PATIENTS WITH GOOD RESPONSE AFTER TRANSPLANT

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Background. Transplant (Tx) remains the gold-standard therapy for younger multiple myeloma (MM) patients (pts). The achievement of good quality responses (CR/VGPR) seems to be a prerequisite for longer progression free survival (PFS). However PFS could be significantly variable among patients with the same type of response (CR/VGPR/PR). No study have been performed until now exploring this aspect. *Aims.* To evaluate any correlation between baseline clinical and laboratory findings and the length of PFS within three groups of patients defined according to the type of response (CR/VGPR/PR) after Tx. *Patients and Methods.* Baseline characteristics of 443 transplanted pts were studied. VAD was used as induction in 370 pts and bortezomib plus dexamethasone in 73 pts. All pts collected stem-cells with chemotherapy plus G-CSF, and conditioning regimen included melphalan 200 mg/m^2. Pts were considered responsive when obtaining at least a PR according to IMWG criteria. The following baseline characteristics were considered for univariate and multivariate analysis: gender, age, Durie and Salmon stage, ISS, number of bone lesions (>3 or ≤3), extramedullary localizations, bone marrow plasmacytosis, haemoglobin level, hypercalcemia, creatinine level, serum and urine monoclonal component, response after induction, type of induction therapy. Type of response, PFS, and PFS in pts grouped according to response after Tx (CR, VGPR, PR) were correlated with categorical and continuous variables through Kaplan-Meyer and Cox methods. *Results.* Taken together all patients had a median follow-up of 44,8 months (1-150 months), a median OS by intention to treat of 92,5 months, and a median PFS after Tx of 24.3 months. 353/443 pts enrolled (80%) were transplanted. At the time of this analysis 25% of the pts (85/339) are free of progression. None of the baseline characteristics showed, in univariate and multivariate analysis, significant correlations either with type of response or with PFS after Tx. Better PFS was registered in pts with CR after Tx (P=0.0001). When pts were grouped according to response after Tx, median PFS were 28.6 (2-106), 22 (6-84), 18 (3-105) months for CR, VGPR, and PR respectively. In pts with CR, high ISS, low haemoglobin level and high plasmocytosis showed a statistically significant shorter PFS. In patients with VGPR, high ISS, low haemoglobin level, hypercalcemia, and high monoclonal component level were significantly correlated with shorter PFS. No significant correlations were found in PR group. *Conclusions.* Our study confirms that Tx allows long-lasting control in younger MM pts, producing in our hands a median overall survival of 92.5 months. Although the achievement of a good quality of response impacts on PFS, there is a great variability among patients with the same type of response after Tx. ISS and hemoglobin level seems to be useful in identifying patients in good response after Tx who could deserve a consolidation or a maintenance to prolong PFS.

0363

THE PROGNOSTIC IMPACT OF FLUORESCENT-IN SITU HYBRIDIZATION (FISH) AND CONVENTIONAL KARYOTYING IN KOREAN MULTIPLE MYELOMA PATIENTS: A RETROSPECTIVE MULTICENTER STUDY

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Background. Cytogenetics and fluorescent-*in situ* hybridization (FISH) are important outcome predictors in multiple myeloma (MM). There were only few small studies that investigated prognostic implication of interphase FISH and/or conventional karyotypes in Korean MM patients. *Aims.* We investigated the incidences and prognostic significances of chromosomal abnormalities detected by interphase FISH and/or conventional karyotyping among Korean MM patients. *Methods.* We collected Korean myeloma patients' data using Korean Multiple Myeloma Registry and performed a retrospective analysis. We analyzed the impact of abnormal karyotypes and FISH anomalies on overall survival. *Results.* From 2000 to 2009, total of 789 newly diagnosed myeloma patients were enrolled in this study. Median age of patients was 62 years. Median overall survival was 68 months, and median follow up of time was 67 months. Among the patients with conventional karyotyping, 28.7% of the patients had abnormal karyotypes. Among the patients with abnormal karyotypes, 46% were hyperdiploid, and 54.0% were non-hyperdiploid. Del13q was found in 26.6% of the patients. Among the patients with interphase-FISH analysis, 23.1% were del13q, followed by t(11;14) (18.5%), t(4;14) (13.9%), del17p (12.0%) and t(14;16) (6.1%). Univariate analyses revealed that abnormal karyotypes (P<.001), del13q (P<.001) by conventional karyotyping, and del13q combined with t(4;14) (P=.019) by interphase-FISH negatively impacted the overall survival. Other genomic aberrations did not affect the overall survival. Clinical parameters that impact on overall survival were infiltration rate of plasma cells in bone marrow, serum beta2-microglobulin, creatinine, low hemoglobin, and low albumin levels. On multivariate analysis, serum creatinine (P=.006), low albumin (P<.001), and del13q by conventional karyotyping (P=.003) were independent risk factors for overall survival. *Summary/Conclusions.* Our results indicate that abnormal karyotypes, del13q by conventional karyotyping, and del13q combined with t(4;14) by interphase-FISH were negative prognostic factors for overall survival in univariate analyses. On multivariate analysis, del13q by conventional karyotyping was independent risk factor for overall survival. In future, prospective trial with laboratory standardization is needed for more reliable results from FISH and conventional karyotyping in MM patients.

0364

NEW STRATEGY FOR ALLOGENEIC PERIPHERAL BLOOD STEM CELL TRANSPLANTATION AFTER REDUCED INTENSITY CONDITIONING IN MULTIPLE MYELOMA: IFM2005-03 STUDY

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Background. The development of new agents with potent anti-tumor activity has considerably improved the survival of multiple Myeloma (MM) patients. However, allografting remains the only available curative treatment particularly for patients at high risk. *Material and Methods.* This is a prospective multicenter study for MM patients of age ≤ 65 years receiving RIC followed by allo-PBSCT after achieving at least a partial response (PR) to auto-transplantation in first line. Patients previously received induction treatment followed by Melphalan 200 mg/m^2. Patients must have an HLA identical donor either from siblings or unrelated 10/10 HLA donors, and at least one of the following poor prognostic factors (PPF): β2 microglobulin >3 mg/L, Del 13, t(4;14) and Del 17p. The conditioning regimen combined Fludarabine 30 mg/m^2/d (d-5→d-1), Busilvex IV 3,2 mg/kg/d (d-4, d-3) and ATG 2,5 mg/kg/d (d-2, d-1). By day 90 post-allograft, patients not in CR received 4 cycles of Velcade 1.3 mg/kg, and after Velcade, if the CR was not achieved, increasing doses of DLI were administered. This analysis included 12 patients, 10 males and 2 females, median age was 46 years [40-60], there were 9 IgG stageA (8κ & 1λ) and 3 IgA (1κ stage B & 2λ stage A). There were 3 patients with 1 PPF, 6 patients with 2 PPF and 3 patients with 3 PPF. *Results.* Seven patients received 4 cycles of VAD (4 patients VAD+DCEP), 2 patients received 4 cycles of Velcade + dex. and 3 patients received other combinations (1PAD, 2VAD then Velcade). After induction, 8 patients were in PR and 4 in stable disease (SD). Patients received auto-HSCT after a median time of 6.6 months [4.5-8.7] from diagnosis. All patients were in PR after auto-HSCT and before allo-PBSCT. The median number of infused CD34+ cells was 6.5×10^6/kg [2.6-13.7] from 4 identical siblings and 8 matched unrelated donors. Sex matching was: F→M:5, F→F:1, M→F: 1 and M→M:5. At D90, 3 patients were in CR and 9 patients received Velcade after absence of CR (2 VGPR and 7 PR). After Velcade, the 2 VGPR evolved to CR and patients in PR became 1 CR, 1 VGPR and 5 remained in PR). One patient in VGPR and 3 in PR received DLI after Velcade, responses were: 1 VGPR and 3 patients progressed. There were 6 acute GVHD (5 grade II and 1 grade III) and 6 chronic GVHD (5 limited and 1 extensive), all GVHD were resolved at the last follow-up. After a median follow-up of 30 months, all patients are alive [100% overall survival (O.S.)], 5 patients are in CR, 1 in VGPR, 5 in PR and one in progression without any active chronic GVHD even after DLI administration. *Conclusions.* We showed very good results due to the better conditioning with the introduction of Busilvex, also the impact of Velcade in eliminating the residual disease. According to these very promising results, we should reconsider the allo-HSCT as a first line treatment for MM especially for patients with PPF using either RIC or standard conditioning depending on age.

0365

OUTCOME OF STAGE B MULTIPLE MYELOMA UNDERGOING HIGH-DOSE THERAPY WITH AUTOTRANSPLANT

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Background. Limited information of the outcome of patients with newly diagnosed multiple myeloma (MM) presenting with serum creatinine > 2 mg/dl (stage B-MM) is available. *Aims.* In the context of an outcomes research project, we analysed the major determinants of treatment response in 71 consecutive, unselected, newly diagnosed stage B-MM patients. *Methods.* Forty-one patients (58%) were enrolled in programs of high dose chemotherapy with autologous stem cell transplant (autoTx), whereas the other 30 (42%) received non-autoTx-based treatments. Treatment allocation was based on patients' age and performance status but not on serum creatinine at diagnosis. *Results.* A the end of first-line therapy, median serum creatinine decreased from 3.75 mg/dL at diagnosis to 1.4 mg/dL, and reverted to values <2 mg/dL in 76% of patients who entered HDT with autoTx programs and in 52% of those who received non-autoTx-based treatments. However, seven of the 15 patients requiring dialysis at diagnosis remained on chronic dialysis. Age > 65 years, anemia and pre-therapy dialysis were associated with a worse outcome. The 71 stage B-MM patients had median overall survival (OS) and event-free survival (EFS) of 21 and 17 months, which decreased to 17 and 14 months for the 30 patients not eligible to HDT with autoTx programs and increased to 25 and 21 months, respectively, for the 41 patients who entered programs of HDT with autoTx. The 28 patients who received at least one autoTx procedure had median OS and EFS of 33 and 29 months, respectively. Thriteen patients received Thalidomide and/or Bortezomib during the induction phase before autoTx procedures. They enjoyed better outcomes (5-yr OS and EFS of 34% and 42%, respectively) compared to the 28 patients who did not receive either drug (5-year OS and EFS of 24% and 12%, respectively). However, these differences did not reach statistical significance. *Summary and Conclusions.* Treatment of stage B-MM improved renal function in the majority of patients, and 60% of them reached at least a partial response. AutoTx procedures were feasible in about 40% of patients, and significantly ameliorated their outcomes. However, when compared to MM patients with a normal renal function, stage B-MM patients showed a higher rate of early mortality and worse outcomes. It is, therefore, necessary to improve treatment in order to enhance response rate and reduce mortality. The incorporation of Thalidomide and, in particular, Bortezomib - which may be safely administered also in patients with severe impairment of renal function - in the first line therapies will help us to reach this goal.

0366

RANDOMIZED PHASE 2 TRIAL OF BORTEZOMIB-DEXAMETHASONE (VD) VERSUS VD PLUS CYCLOPHOSPHAMIDE OR LENALIDOMIDE IN MYELOMA PATIENTS ACHIEVING STABLE DISEASE AFTER 4 CYCLES OF VD AS SECOND-LINE TREATMENT

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Background. Bortezomib plus dexamethasone (VD) is known to be effective and well tolerated in patients with multiple myeloma (MM). As demonstrated in the frontline setting, the addition of cyclophosphamide (VCD) or lenalidomide (VRD) may lead to improved efficacy, but may be associated with increased toxicities; however, few studies have prospectively assessed VD as second-line therapy. *Aims.* This randomized, open-label, parallel-group, phase 2 study in patients with relapsed or refractory MM was designed to evaluate the safety and efficacy of an additional 4 cycles of bortezomib based therapy following 4 initial cycles of VD (Vel 1.3 mg/m^2 IV on days 1, 4, 8, and 11, and Dex 20 mg PO on days 1, 2, 4, 5, 8, 9, 11, and 12) totalling a maximum of 8 treatment cycles. *Methods.* Patients achieving at least partial response (PR) received 4 cycles of VD; patients with SD were randomised to 4 cycles of VD, VCD or VRD. Patients with progressive disease (PD) discontinued treatment. Bortezomib-naïve patients aged ≥18 years and with a KPS ≥60 were eligible; patients with grade ≥2 peripheral neuropathy (PN) were ineligible. *Results.* A total of 183 patients were enrolled; 32 had not received therapy and were excluded from the safety population (N=151). The median age at baseline was 63 years (range 34-86), 49.6% were male, 20.3% had KPS ≤70; median time from prior therapy was 14.9 months. In 92 patients, efficacy was evaluable after cycle 4. Overall response rate of 83.7% (including 10.9% CR, 25% VGPR, 47.8% PR) was observed. A further 15 (16.3%) patients had SD; 17 patients were randomized to sequential therapy. Median time to first and best response was 43 and 64 days, respectively. The glomerular filtration rate (GFR) results are shown in the Table. 54% of patients experienced grade 3/4 AEs and 36% had serious AEs. The most common grade 3/4 AEs included thrombocytopenia (13%), anemia (8%), and pneumonia (5%). AEs resulting in dose reductions/treatment stop were seen in 38%/21% of patients. Incidence of sensory PN and PN was 34% (7% grade 3/4); 76% of PN events were reversible, with resolution in 33.5% and improvement in 42.5% in a median of 241 and 55 days, respectively. *Conclusions.* This study further demonstrates that VD treat-

ment is effective and well tolerated with less than 10% of patients needing sequential therapy. Overall renal function was shown to improve with treatment. VD represents a feasible, active treatment option for patients with relapsed MM. Acknowledgments: The authors would like to thank all of the MMY-2045 study investigators.

Table. Renal function (as measured by GFR)*.

Renal function group, n†	
Baseline	121
GFR <15 mL/min	3
15–<30 mL/min	9
30–<60 mL/min	46
≥60 mL/min	63
Renal improvement by at least 1 grade, n (MM response)	12
<15 to 15–<30 mL/min	1 (1 CR)
15–<30 to 30–<60 mL/min	2 (1 VGPR, 1 PR)
30–<60 to ≥60 mL/min	9 (1 CR, 1 VGPR, 4 PR, 3 SD)

*1 patient only had a baseline GFR measurement and was not included in the renal analysis
†1 patient had no baseline GFR measurement

0367

A PHASE III STUDY COMPARING THALIDOMIDE/CYCLOPHOSPHAMIDE/ DEXA VS THALIDOMIDE/DEXA VS THALIDOMIDE/MELPHALAN/PREDNISONE IN DE NOVO MULTIPLE MYELOMA PATIENTS NOT ELIGIBLE FOR ASCT: PRELIMINARY ANALYSIS

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Background. Thalidomide (Thal) is a pro-apoptotic, immunoregulatory and antiangiogenic agent for MM. Although it is used since the 90's, the optimal combined treatment remains inconclusive. *Aims.* This is a preliminary analysis of the first Latin American prospective open-label study comparing three different combinations with Thal for MM patients not eligible for autologous SCT. *Patients and Methods*. Eligible patients were randomized to one of the three arms and received nine 28-days induction cycles. All patients received Thal(100-200 mg/d) and one of the following: A- Cyclo (50 mg/d)+ Dexa (40 mg/D1-4, 15-18 in cycles 1 and 2, D1-4 in cycles 3 to 9), B- Dexa (40 mg/D1-4, 9-12, 17-20 in cycles 1,3,5,7 and 9, D1-4 in even cycles) or C- Mel (4 mg/m^2/7d)+ Pred (40 mg/m^2/7d). Thereafter, they were randomized to maintenance treatment until progression disease or unacceptable toxicity, as follows: A1-Thal (100 mg/d)+Pred (50 mg/each other day) or B1-(Thal 100 mg/d). All patients received aspirin as thromboprophylaxis and sulphatrimetroprim as prophylaxis for infection. If indicated, they received biphosphonate monthly for 24 months, and then quarterly. Before study initiation, informed consent was obtained from patients and evaluation was performed at the end of each cycle. The primary endpoint was response rate and response duration. The secondary endpoints were safety, OS and PFS. IMWG index was utilized to analyze response criteria. *Results.* Enrolment started on February 2007, and a total of 54 patients have been included. Median age at randomization was 71 (56-84) years-old, and 57% of patients were female. Durie-Salmon stages were 10% II, 84% III and ISS stages were 43% II and 33% III. Fifty-one per cent of patients presented IgG monoclonal component. After 3 cycles, 63% of patients had total objective response, 6.3% had CR, 14.9% had VGPR, and 42.5% had PR. After six cycles, total objective response was 70.2%, CR=10.6%, VGPR=19.2% and PR=40.4%. Up to present, 44.7% of patients have concluded 9 cycles with a total objective response of 90%, CR=19%, VGPR=19%, and PR=62%. The Table 1 shows the response rate of each arm and cycle (until cycle six). Progression disease was observed in one patient. Nine deaths occurred until now and 7 during induction phase, 5 of them within the first 4 months. Infection was the major cause of death. During induction, 42% of patients experienced grade 3/4 toxicity, including grade 3/4 neutropenia or infection (4 patients), neuropathy (8), and venous thrombosis (1). Dose reduction was required for 4 patients due to toxicity, but no discontinuation has occurred. The median follow-up for all patients was 226 days (11-1010) and 80% of patients are still alive. *Discussion.* This preliminary analysis suggests that Thal combinations are effective in MM patient treatment. The alkylating combination with thalidomide has demonstrated better objective response than Thal+Dexa. Toxicity was acceptable in all arms. Performance status could justify the initial mortality rate. With more patients included in this study, probably we will get to define the best Thalidomide combination for MM patients not eligible for ASCT.

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Table 1.

INDUCTION	CYCLE 1		CYCLE 2		CYCLE 3		CYCLE 4		CYCLE 5		CYCLE 6	
ARM	PATIENT NUMBER	ORR	PATIENT NUMBER	ORR	PATIENT NUMBER	ORR	PATIENT NUMBER	ORR	PATIENT NUMBER	ORR	PATIENT NUMBER	ORR
A	17	7 (41%)	16	13 (81%)	16	13 (81%)	14	11 (79%)	12	11 (92%)	11	10 (91%)
B	13	3 (23%)	11	5 (45%)	11	7 (64%)	9	5 (56%)	7	2 (29%)	6	4 (67%)
C	16	4 (25%)	14	7 (50%)	14	9 (64%)	14	9 (64%)	14	10 (71%)	14	10 (71%)
OVERALL RESPONSE	46	14 (30%)	41	25 (61%)	41	29 (71%)	37	25 (68%)	33	23 (70%)	31	24 (77%)

* ORR = Total Objective Response (CR/VGPR/PR)

0368

SEQUENTIAL VAD (VINCRISTINE, ADRIAMYCIN, DEXAMETHASONE) AND VTD (BORTEZOMIB, THALIDOMIDE, DEXAMETHASONE) INDUCTION FOLLOWED BY HIGH-DOSE THERAPY WITH AUTOLOGOUS STEM CELL TRANSPLANTATION AND MAINTENANCE TREATMENT WITH BORTEZOMIB FOR NEWLY DIAGNOSED MULTIPLE MYELOMA (A PHASE II TRIAL)

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Background. Incorporation of novel agents has resulted in an improved response rate and reduced side effects in multiple myeloma. This has suggested the possibility of using novel agents as induction chemotherapy in patients with newly diagnosed multiple myeloma. *Aims.* We studied efficacy and safety of sequential VAD and VTD as induction chemotherapy. *Methods.* The patients were planned to receive 2 cycles of VAD and VTD. Bortezomib as a maintenance treatment was administered weekly x 4 times every 6 weeks for 4 cycles after ASCT. *Results.* A total of 71 patients were enrolled, and efficacy could be assessed in 59 patients. After 2 cycles of VAD, the RR was 67.8%. After VTD, the RR was more increased to 96.6% (CR and near CR: 27.1%). Especially, 7 patients with poor prognostic cytogenetics all responded after VTD. Autologous stem cells were successfully collected in all 52 patients with a median CD34$^+$ count of 7.12×10^6/kg (range, 1.94-44.7 ×10^6/kg), except one patient. After ASCT, 30 patients completed bortezomib maintenance, and the rate of CR and near CR was 70%. The median follow-up duration was 34.6 months, and the median time to response was 1.6 months. The median TTP and OS were not reached. *Conclusions.* The sequential VAD and VTD induction therapy in patients with newly diagnosed multiple myeloma was effective with tolerable toxicity profiles and it did not prejudice stem cell collection. VTD could have contributed to the increased RR and minimized the side effects as an induction therapy, and maintenance therapy with bortezomib maintained a very good overall response rate.

0369

EFFICACY AND SAFETY OF WEEKLY INFUSION BORTEZOMIB IN ELDERLY NEWLY DIAGNOSED MULTIPLE MYELOMA PATIENTS

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Background. Frequently observed toxicities associated with bortezomib-based therapy include peripheral neuropathy (PN), thrombocytopenia and gastrointestinal dysfunction. Bortezomib related PN is a common complication (≥G3 22%), particularly among elderly patients, it is dose related and reversible in most cases with treatment interruption and dose modifications. *Aims.* To evaluate the efficacy, the safety and the risk factors of bortezomib related adverse events, particularly PN, in patients with newly diagnosed myeloma treated with different schedules of bortezomib, in a phase III randomized study of bortezomib-melphalan- prednisone-thalidomide followed by continuous treatment with bortezomib-thalidomide (VMPT-VT) versus bortezomib-melphalan-prednisone (VMP). *Methods.* Between May, 2005 and January, 2009, 511 patients older than 65 years were randomly assigned to VMPT-VT or VMP. In the VMPT-VT arm patients received bortezomib 1.3 mg/m^2 days 1,4,8,11,22,25,29,32 in cycles 1-4 and days 1,8,22,29 in cycles 5-9; melphalan 9 mg/m^2 plus prednisone 60 mg/m^2 days 1-4 and thalidomide 50 mg days 1-42 for nine-6-week cycles, followed by VT therapy (bortezomib 1.3 mg/m^2 days 1, 15, thalidomide 50 mg/day). In the VMP arm patients received the same schedule without any further therapy. To evaluate if the treatment regimens could be further optimized by decreasing the toxicity while maintaining efficacy, in March 2007 the protocol was amended and both bortezomib schedules were reduced to once weekly infusion. *Results.* 503 patients were evaluable for safety and efficacy, 369 patients were treated with once-weekly infusion of bortezomib and 134 with twice-weekly infusion. The response rate was similar in once-weekly group as compared with twice-weekly group: ≥PR in 85% vs 86% of patients (P=.73), with a VGPR rate of 55% vs 54% (P=.84) and CR of 29% vs 35% (P=.23), respectively. There aren't differences between once-weekly and twice-weekly patients in the 3-y PFS (50% vs 47%, P=.99) and 3-y OS (88% vs 89%, P=.54). Regarding toxicity, non-hematologic grade 3 to 4 adverse events were reported in 36% of once-weekly patients and in 51% of twice-weekly patients: the reduction was significant (P=.003) and was mainly related to grade 3 or 4 sensory PN that was significantly reduced from 16% to 3% (P<.001), without any significant difference between VMPT-VT and VMP groups. In multivariate analysis the only predictive factor of lower incidence of PN (P<.0001) was the reduction of bortezomib infusion from twice to once weekly. The proportion of patients who required treatment interruption for PN was 5% in the once-weekly patients and 15% in the twice-weekly patients (P<.0001). Similarly, the proportion of patients who required bortezomib dose reduction was 24% and 54%, (P<.0001), respectively. This improvement in the discontinuation and dose reduction rate prolonged the time on therapy: the cumulative delivered bortezomib dose compared with planned dose was 40.0 mg/m^2 (46.8 mg/m^2) in once-weekly patients and 41.0 mg/m^2 (67.6 mg/m^2) in twice-weekly patients. *Conclusions.* The weekly infusion of bortezomib significantly decreased the incidence of severe PN, discontinuation rate, dose reduction rate and prolonged the time on therapy. This unprecedented improvement of safety came with no substantial reduction in terms of efficacy.

0370

OUTCOME OF ELDERLY PATIENTS 70 YEARS AND OLDER WITH NEWLY DIAGNOSED MYELOMA IN THE ECOG RANDOMIZED TRIAL OF LENALIDOMIDE/HIGH-DOSE DEXAMETHASONE (RD) VERSUS LENALIDOMIDE/LOW-DOSE DEXAMETHASONE (RD)

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Aim. Lenalidomide and dexamethasone is increasingly being used as frontline therapy in myeloma in elderly patients, but data on its efficacy in this cohort compared with other standard regimens is not known. The goal of this study was to determine and describe the safety and efficacy of this combination in the subset of elderly patients 70 years of age and older treated on the ECOG phase III trial of lenalidomide plus high (standard) dose dex (RD) versus lenalidomide plus low dose dex (Rd) in newly diagnosed myeloma (MM). *Methods.* The ECOG E4A03 trial enrolled 445 adult patients with newly diagnosed myeloma (median age, 65 yrs) with no age restriction, and results have been published (Lancet Oncol 2010;11:29-37). For this analysis, pts 70 years and older treated on this trial were studied. Pts in the RD arm received lenalidomide 25 mg/day PO days 1-21 every 28 days plus dex 40 mg days 1-4, 9-12, and 17-20 PO every 28 days; pts in the Rd arm received lenalidomide at the same dose plus dex 40 mg days 1, 8, 15, and 22 PO every 28 days. The primary endpoint for this analysis was overall survival at 3 years. Response and progression free survival (PFS) were studied as additional endpoints. All analyses were performed on intent to treat basis. *Results.* 147 patients ages 70 years and older (76 randomized to RD and 71 randomized to Rd) were studied. 15 patients (10%) went onto SCT. Median follow-up time is 36 months. Treatment duration was 3.9 mos (RD) and 9.7 mos (Rd), P=<0.001. The overall response rate (partial response or better) was 74% (RD=75% and Rd=74%), including 45% with very good partial response or better (RD=42% and Rd=48%). Median PFS was 22 months with Rd, and 16 months with RD, P=0.11. The 3-year overall survival rate was 66%. Overall survival in this cohort of patients 70 and older was significantly superior with lenalidomide plus low dose dex, P=0.03; 3-year overall survival 73% (Rd) versus 61% (RD). Overall survival differences persisted when survival was analyzed after excluding the 15 pts who underwent SCT (P=0.02), with corresponding 3-year survival rates of 70% with Rd and 57% with RD. Forty-eight patients have died; 23 due to disease progression, 6 cardiac causes, 6 unknown, 5 infection, 3 thrombosis/embolism, and the rest due to other causes. The rate of grade 3 or higher non-hematologic toxicities was 59% with Rd, and 78% with RD. This included deep vein thrombosis rates of 20% (Rd) and 30% (RD); corresponding rates for infections were 10% and 20%, respectively. *Conclusions.* Lenalidomide plus low-dose dexamethasone (Rd) appears to be safe and effective in elderly patients 70 years of age and older with newly diagnosed multiple myeloma, with a 3-year overall survival rate of 73%. This non-alkylator-containing oral regimen merits comparison with standard regimens such as bortezomib, melphalan, prednisone.

0371

PHASE II STUDY OF BORTEZOMIB, THALIDOMIDE, AND DEXAMETHASONE +/- CYCLOPHOSPHAMIDE AS INDUCTION THERAPY IN PREVIOUSLY UNTREATED MULTIPLE MYELOMA (MM): SAFETY AND ACTIVITY INCLUDING EVALUATION OF MRD

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Background. Bortezomib plus thalidomide-dexamethasone (VTD) has demonstrated substantial activity as induction therapy in MM. Bortezomib has also shown activity in combination with cyclophosphamide-dexamethasone in treatment-naïve MM patients. While 4-drug regimens may build on efficacy of three-drug combinations, they may also be associated with increased toxicity. *Aims.* To determine whether addition of cyclophosphamide to VTD (VTDC) increases efficacy, both post-induction and following HDT-ASCT, without compromising safety. *Methods.* This randomized, phase 2, non-comparative study enrolled 98 previously-untreated, transplant-eligible MM patients (aged 18-70

years) with measurable disease. Patients received four 21-day cycles of VTD (bortezomib 1.3 mg/m^2 [days 1, 4, 8, 11]; thalidomide 100 mg daily; dexamethasone 40 mg [days 1-4, 9-12];n=49) or VTDC (VTD plus cyclophosphamide 400 mg/m^2 IV [days 1, 8];n=49), as induction therapy prior to HDT-ASCT. Patients could receive an additional 4 cycles if they became transplant-ineligible or had complete response (CR) post-induction. Responses were categorized using modified IMWG Uniform Response Criteria (stringent CR replaced by CR with normalized serum κ:λ ratio [CRflc]).The primary endpoint was combined CR rate (CRflc+CR+near-CR) post-induction. Secondary endpoints included combined CR rate post-HDT-ASCT, overall response rate (ORR: ≥partial response) post-induction and post-HDT-ASCT, time to progression (TTP), overall survival (OS), quality of life (QoL), and safety. Adverse events (AEs) were graded using NCI CTCAE v3.0. Bone marrow samples taken during suspected CR were used to assess minimal residual disease (MRD) status by flow cytometry, which may be more prognostically relevant than conventional response criteria. *Results.* In the VTD/VTDC arms, respectively, median age was 57/58 years and 18%/24% had Karnofsky performance-status ≤70%. Four VTDC patients received additional cycles of treatment. To date, 48 VTD and 35 VTDC patients have undergone HDT-ASCT. Median CD34$^+$ stem cell yields were 8.16 (VTD; n=48) and 8.13 (VTDC; n=40)×10^6/kg. Post-induction and post-HDT-ASCT response rates are shown in the table. Median TTP and OS have not been reached (median follow-up: 9.7/9.9 months). Estimated 1-year survival rate was 94% in each arm. MRD was evaluated in 23 patients in each arm during/post-induction; 17 (VTD) and 13 (VTDC) were MRD-negative. Most of these MRD-negative patients achieved combined CR (VDT n=15; VTDC n=10; per above criteria), apart from 2 VTD and 3 VTDC patients who had ≤VGPR. Post-transplant (samples collected after 40-269 days), 9 (VDT) and 3 (VTDC) additional patients became MRD-negative (all CR), thus a total of 26 (VTD; 53%) and 16 (VTDC; 33%) patients provided an MRD-negative sample during the study. Most patients experienced ≥1 treatment-emergent AE (VTD 98%, VTDC 96%), including 47% and 59% with grade ≥3 AEs. Most common non-hematologic grade ≥3 AEs (VTD/VTDC) included constipation (6%/2%), fatigue (2%/8%), and peripheral-neuropathy (10%/8%). Grade 3/4 hematologic toxicities (laboratory evaluation; VTD/VTDC) included lymphopenia (39%/78%), anemia (8%/18%), neutropenia (14%/18%), and thrombocytopenia (6%/6%). By EQ-5D and EORTC-QLQC30 QoL assessments, both treatments increased patient perceptions of health and well-being over the long-term. Summary: VTD and VTDC are both highly active induction regimens, resulting in high CR rates and MRD-negative rates. Depth of response improved post-HDT-ASCT. Although efficacy profiles were similar, rates of toxicity were higher with VTDC than VTD.

Table 1.

	N*	Combined CR (M-protein confirmed), %	Combined CR (bone marrow confirmed), %	CR^{flc}, %	≥VGPR %	ORR %
Best response post induction						
VTD	49	51	45	27	69	100
VTDC	48	44	35	27	69	96
Best response post HDT-ASCT						
VTD	38	76	58	39	87	100
VTDC	27	78	48	33	85	100

Median time to response	Time to first response, days	Time to combined CR, days
VTD	22	97
VTDC	23	139

*Response-evaluable patients
FLC = serum free light chain; VGPR = very good partial response

0372

WITHDRAWN BY AUTHOR

Myeloma and other monoclonal gammopathies - Clinical 2

0373

THE INTERNATIONAL STAGING SYSTEM FOR MULTIPLE MYELOMA REMAINS RELEVANT IN THE NOVEL AGENT ERA ONLY IN PATIENTS RELAPSING GREATER THAN 12 MONTHS FROM HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. The international staging system (ISS) for multiple myeloma (MM) has been validated as a prognostic tool based on biochemical parameters at diagnosis. At present, there is no validated model to predict response to therapy at relapse. In British Columbia the standard of care for multiple myeloma (MM) includes upfront hematopoietic stem cell transplantation (HSCT) in eligible patients. Since 2004 thalidomide, bortezomib and lenalidomide, the so called novel agents (NA) have been available for use in the relapsed setting. *Aims.* To examine whether the ISS remains relevant in predicting overall survival (OS), progression free survival (PFS) and post relapse survival (PRS) in the NA era. Whether early relapse (<12 m post SCT) further identifies high-risk disease.

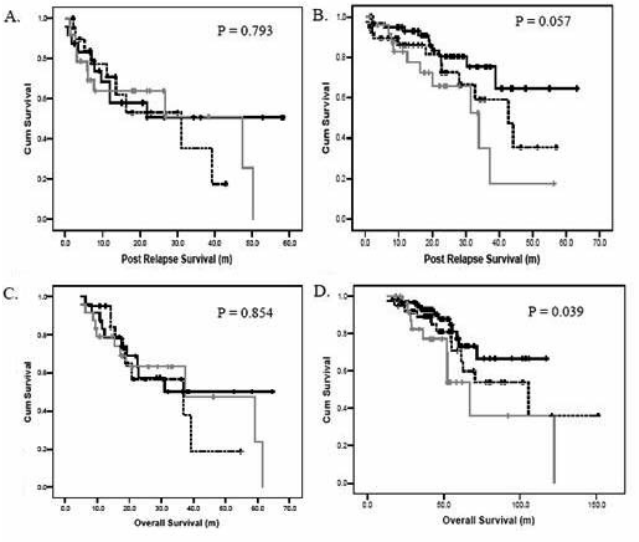

Figure 1: Kaplan-Meier curve demonstrating OA and PRS (early relapse (A and C) and late relapse (B and D)) based on ISS stage and timing of relapse. ISS 1 (—), ISS 2 (▪▪▪), ISS 3 (___).

Methods. We reviewed 230 patients with MM in our prospectively collected database treated with upfront HSCT who relapsed post January 1st, 2004. After this point NA became widely available for relapsed patients defining the NA era at our centre. 195 had complete ISS data. OS and PFS were measured in months (m) from time of HSCT. PRS was measured from the time of relapse after first SCT. The cohort was further split based on timing of relapse and assessed in terms of the ISS stages. *Results.* Median OS, PFS and PRS by ISS stage are summarized in Table 1. When separated by early and late relapse both OS (37.5 m vs. 105.5 m; P<0.001) and PRS (31.0 m vs. 42.8m; P=0.003) are significantly different. When the ISS was applied to the early and late relapsing cohort to predict PRS and OS it remained relevant only for late relapsing patients (Figure 1). This significance was also seen for OS (not shown). Lastly, patients who relapsed early had similar PRS (31.0 m vs. 34.0m; P=0.50) when compared with ISS 3 patients. *Conclusions.* From our data the ISS remains a useful tool to predict OS and PRS for patients with MM relapsing in the NA era. A subset of patients with aggressive disease will be under recognized by characteristics at diagnosis. Our data supports that early relapse is an important clinical prognostic mark-

er identifying high risk patients irrespective of baseline features defined by the ISS. Compared to those with ISS 3 disease, patients with early relapse had a similar PRS identifying a high risk cohort of patients that may warrant more aggressive post relapse therapy and consideration of enrollment in clinical trials.

0374

ELOTUZUMAB IN COMBINATION WITH BORTEZOMIB IN PATIENTS WITH RELAPSED/REFRACTORY MULTIPLE MYELOMA WITH ONE TO THREE PRIOR THERAPIES: A PHASE 1 STUDY

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Background. Elotuzumab is a humanized antibody against CS1, a cell surface glycoprotein that is highly and uniformly expressed in multiple myeloma (MM). In mouse xenograft models of MM, elotuzumab demonstrated significantly enhanced antitumor activity when combined with bortezomib compared to bortezomib alone (Tai et al Blood 2008). *Aims.* This phase 1 study was designed to determine maximum tolerated dose (MTD), safety, pharmacokinetics, and response of elotuzumab in combination with bortezomib in patients (pts) with relapsed/refractory MM. *Methods.* Four escalating doses of elotuzumab (2.5-20 mg/kg) were administered on day 1 and day 11 and bortezomib (1.3 mg/m^2) on days 1, 4, 8, and 11 of each 21-day cycle. Pts with ≥ stable disease after 4 cycles continued treatment until progression. Entry criteria included age ≥18 years; confirmed MM diagnosis with 1 to 3 prior therapies (prior bortezomib use was allowed); and measurable M-protein in serum and/or urine. *Results.* Twenty-eight pts with relapsed and relapsed and refractory MM have been treated; 3 each at 2.5, 5, and 10 mg/kg and 19 at 20 mg/kg of elotuzumab, respectively. Twenty-six pts have completed data analysis, with median 3.4 years from initial MM diagnosis and median 2 prior lines of therapy. Eight pts (31%) had previously been treated with bortezomib and 9 (35%) were refractory to their last MM therapy. No dose-limiting toxicity was observed during the first treatment cycle and the MTD was not reached. Twelve serious adverse events (SAEs) were reported in 9 pts with 2 grade (G) 3 events, including chest pain and gastroenteritis, assessed as elotuzumab-related. Unrelated SAEs included G4 hypercalcemia and metabolic encephalopathy, G3 myocardial infarction, sepsis, vomiting (all 1 pt), pneumonia (2 pts), G2 dehydration, G1 ileus, and G1 incoherence. Most common adverse events included fatigue, diarrhoea, nausea, thrombocytopenia, constipation, anaemia, peripheral neuropathy, lymphopenia, neutropenia, and leukopenia. Best response (≥ MR) by the combined European Group for Blood and Marrow Transplantation and uniform criteria in 20 response evaluable pts with ≥2 treatment cycles was 60% including 40% ≥ PR. Median time-to-progression (TTP) is currently 9.6 months. Preliminary analysis of bone marrow plasma cells indicated that > PR correlates well with complete or near complete saturation of CS1 sites on MM by elotuzumab. *Summary/Conclusions.* The combination of elotuzumab with bortezomib has a manageable safety profile and encouraging activity in relapsed and refractory MM with ≥ MR in 60% of pts and a median TTP of 9.6 months. Updated safety and efficacy data will be presented at the meeting.

0375

THE IMPROVED SURVIVAL OF PATIENTS WITH MULTIPLE MYELOMA AFTER THE INTRODUCTION OF NOVEL AGENTS IS IRRESPECTIVE OF ADVANCED ISS, RENAL IMPAIRMENT OR INCREASED LDH: AN ANALYSIS ON 1501 PATIENTS

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Background. The introduction of novel anti-myeloma agents, such as thalidomide, bortezomib and lenalidomide has led to a substantial improvement in the overall survival (OS) of patients with multiple myeloma (MM) during the last decade. However, there is limited information for OS of specific subgroups of MM patients after the introduction of novel agents. *Aim.* The purpose of our analysis was to evaluate OS in a large number of unselected MM patients who were treated throughout Greece to define subsets of patients who were benefited the most from the administration of novel agents. *Patients/Methods.* We compared the outcome of two patient cohorts who started treatment before or after the introduction of the first novel drug, thalidomide; from 1/1/1985 till 30/9/2009, 1501 patients (841M/660F, median age 72 years) were entered into the database of the Greek Myeloma Study Group: 859 patients started treatment before 31/12/1999 (Group A) and 642 patients after 1/1/2000 (Group B), when thalidomide became available in Greece. *Results.* Patients in group A were younger (P<0.001), had less often hypercalcemia (P=0.03) and had less often advanced ISS stage (P=0.014). The majority of patients in Group B were treated upfront with novel-drug based regimens compared to only 2 (0.2%) in Group A (P<0.0001). The median follow-up of all patients was 81 months. Despite more favorable characteristics of patients in Group A, their median overall survival was 36 months compared to 50 months of patients in Group B (P<0.0001). Improved survival was observed in all ISS stages of Group B compared with the respective OS of group A: median OS for ISS-1, ISS-2 and ISS-3 was 59, 36 and 18 months, respectively for patients of group A, and 96, 49 and 32 months, respectively for patients of group B (P=0.006, 0.001 and <0.0001, for the three comparisons, respectively). The administration of novel agents improved median OS in patients with renal impairment (defined as serum creatinine ≥2 mg/dL): 28 months versus 20 months in group A (P=0.012). Similarly, the administration of novel agents improved median OS in patients with high LDH (>300 IU/L): 20 months versus 14 months in group A (P=0.048). The greatest benefit in OS by novel agents was observed in patients ≤65 years of age in whom the median OS has improved more than 2-fold in group B (96 months versus 42 months in group A, P<0.0001). The survival benefit with novel agents was also observed in patients aged 65-70 years (median OS: 60 months in group B versus 30 months in group A, P=0.001). However, in patients above the age of 70 years the administration of novel agents did not produce a significant survival advantage [median OS: 33 months in group B (n=248 patients) versus 27 months in group A (n=247 patients), P=0.001]. *Summary/Conclusions.* The introduction of novel drugs has significantly improved the outcome of MM even in patients with ISS-3, renal impairment or high LDH. The survival benefit was more pronounced in patients ≤70 years of age; thus the management of older patients needs to be improved.

0376

OUTCOME OF PATIENTS WITH MYELOMA RELAPSING AFTER IMID AND BORTEZOMIB THERAPY: A MULTICENTER STUDY FROM THE INTERNATIONAL MYELOMA FOUNDATION WORKING GROUP

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Background. Patients (pts) with multiple myeloma (MM) have increased treatment options with the advent of novel agents such as the IMiDs and bortezomib. However, the disease invariably relapses following use of these drugs and ongoing efforts are targeted to developing new agents for MM. Furthermore, the natural history of MM following the failure of these novel agents is not well understood and is critical for evaluating the potential impact of upcoming experimental therapies on outcome. *Methods.* We designed a multicenter, retrospec-

tive study that enrolled 291 pts with relapsed MM, from 14 sites (107 pts from US; 115 from Europe; and 69 from Asia). Pts were refractory to Bz, defined as no response to prior Bz-containing regimen or disease progression within 60 days of a Bz-containing regimen. Pts were also relapsed, refractory, intolerant, and/or ineligible, to treatment with an IMiD. Clinical and laboratory data from diagnosis and individual relapses were collected. The date pts satisfied the above entry criteria were defined as time zero (T0). *Results.* Median age at diagnosis was 58 years (range 30, 85) and median time from diagnosis to T0 was 3.3 years (0.18, 18.7). Median time to T0 was 4.0, 3.3, and 2.8 for US, European and Asian groups (P=0.006). Following T0, 216 (74%) pts had a treatment recorded, with 93%, 63% and 65% in the US, European and Asian groups receiving therapy respectively. Various regimens were utilized after T0 (median=1; range 0-8), with the US group receiving more regimens following T_0 compared to the other two. Minimal response or better was seen to first salvage regimen after T0 in 73 (34%) of pts; 29%, 43% and 29% in the US, Europe and Asian groups respectively. Median overall survival (OS) and event free survival from T0 were 8 mos (95% CI; 6,10) and 5 mos (95% CI; 4,5) for the entire group. Median OS was significantly longer for the US group (13 mos) compared to the European (7 mos) and the Asian (8 mos) groups(P=0.006). EFS was comparable across the groups. There were 22, 13 and 11% of pts who had a transplant after T0 in the three groups respectively. Excluding these pts, median OS was comparable across the three groups, (P=0.18) . In multivariate analysis examining factors affecting OS from T0, serum albumin, B2M and age at diagnosis were prognostically significant (P<0.05). *Conclusions.* This study confirms the poor outcome of pts once they relapse and become refractory to existing novel agents. Median OS of 8 mos and EFS of 5 mos can be used for comparing the results of ongoing clinical trials in this population. The study also serves to highlight the differences in treatment patterns, including the use of transplant later in the course of disease in US pts, as well as the trend towards the use of multiple salvage regimens, with many in the context of clinical trials.

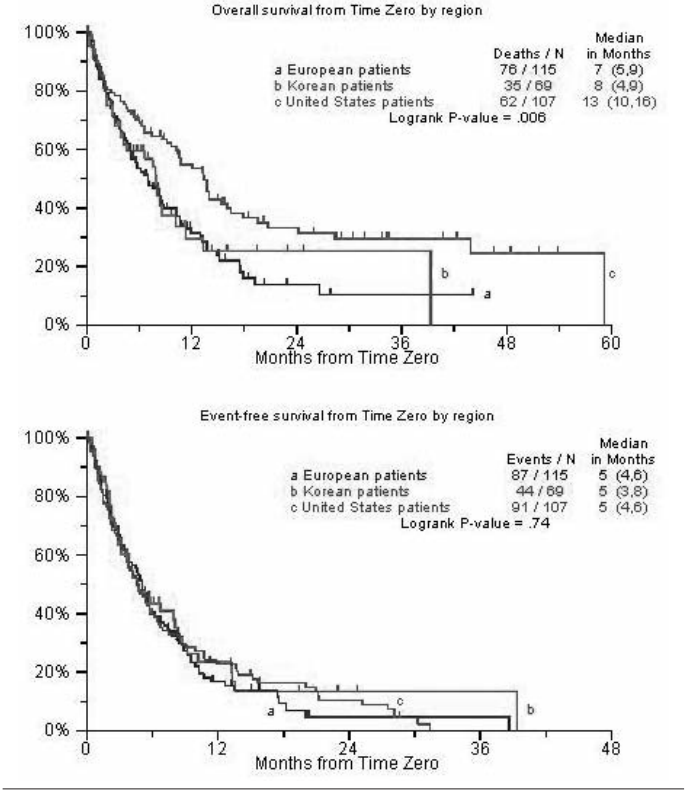

Figure. Kaplan Meier graph for outcomes from time

0377

EFFICACY AND SAFETY OF RETREATMENT WITH BORTEZOMIB IN PATIENTS WITH MULTIPLE MYELOMA: INTERIM RESULTS FROM RETRIEVE, A PROSPECTIVE INTERNATIONAL PHASE 2 STUDY

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Background. Bortezomib retreatment has been demonstrated to be efficacious and well tolerated in patients with relapsed multiple myeloma (MM) in a number of retrospective studies and a small prospective phase 4 study. *Aims.* RETRIEVE is a large, prospective, international, open-label phase 2 study that aims to confirm the efficacy and safety of retreatment with bortezomib in MM patients who had previously responded (at least partial response [PR]) to bortezomib-based therapy as their most recent prior treatment. *Methods.* Patients had to have previously tolerated bortezomib alone or in combination and have had a treatment-free interval (TFI; time from last dose of initial bortezomib treatment to first dose of bortezomib retreatment) of ≥6 months. Patients received bortezomib at the last dose (1.0 or 1.3 mg/m^2) tolerated during initial treatment on days 1, 4, 8, and 11 for up to eight 21-day cycles, either alone or in combination with dexamethasone at the investigator's discretion. Response was assessed by European Group for Blood and Marrow Transplantation (EBMT) criteria every 6 weeks during treatment and then every 2 months until disease progression. Adverse events (AEs) were graded according to NCI CTCAE v3.0. *Results.* A total of 130 patients received at least 1 dose of bortezomib retreatment and were included in the safety population. Patients had a median age of 67 years, median time from diagnosis of MM was 4.5 years (range 0-14 years); and median number of prior therapies was 2; 15, 80, 23, and 12 patients had received 1, 2, 3, and ≥4 prior lines of therapy (including initial bortezomib therapy), respectively. Patients received a median 7.0 (range 1-8) cycles of bortezomib retreatment (23% of patients completed all 8 cycles); 72% of patients received concomitant dexamethasone. In 126 patients, the overall response rate (ORR; complete response [CR]+PR) to retreatment by best confirmed response was 39.7%; in addition, 18.3% of patients achieved minimal response (MR), to demonstrate a CR+PR+MR rate of 58%. Best response to initial bortezomib treatment was CR in 26% and PR in 74% of patients. In patients who achieved a CR or PR to initial bortezomib treatment, the ORR to bortezomib retreatment was 62.5% and 52.1%, respectively. 97.7% of patients experienced a treatment-emergent AE; 53.8% experienced a grade 3/4 AE, and 31.5% experienced a serious AE. The most common grade 3/4 AEs included thrombocytopenia (24%), neutropenia (7%), diarrhea (5.4%), and pneumonia (1.5%). The overall incidence of neuropathy was 39%, including 8.5% grade 3. Most peripheral neuropathy events were reversible; with 61% resolving within a median of 39 days. *Conclusions.* These results confirm that bortezomib retreatment is a well-tolerated, feasible and active therapeutic option in MM patients without evidence of cumulative toxicity. Acknowledgments: The authors would like to thank all of the RETRIEVE study investigators.

0378

OUTCOME OF AUTOLOGOUS STEM CELLS TRANSPLANTATION (ASCT) IN COMPARISON WITH NEW DRUG-BASED REGIMENS AS SALVAGE TREATMENT AFTER FIRST LINE THERAPY WITH SINGLE OR TANDEM ASCT IN MULTIPLE MYELOMA PATIENTS

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Background. Multiple Myeloma (MM) remains still incurable despite the use of several new therapeutical options. High-dose melphalan+ASCT represents the standard therapy for eligible patients, although new trials are currently addressing the usefulness of ASCT in the era of novel drugs. Therapeutic options for patients relapsing after

ASCT include novel biologic agents, traditional chemotherapy and/or ASCT, but few studies about ASCT efficacy and advantage in relapse setting after single/tandem ASCT are actually available. *Aim*. We have therefore analyzed an uniform cohort of patients receiving as second line therapy new drug-based regimens or standard chemotherapy with or without further consolidation with ASCT. *Methods.* In 161 MM patients treated between 1997 and 2008 with HD-Mel and ASCT as first line therapy, relapse has occurred in 105 (65%). Females were 70 (43%), males 91 (57%), median age was 61 (range 34-75). As induction therapy before ASCT, 130 (81%) received VAD- and 31 (19%) thalidomide/bortezomib-based regimens. 85 patients (53%) received single ASCT and 76 double (47%). Second-line therapy was given to 98/105pts: 60pts (61%) received thalidomide/bortezomib-based regimens (TB group), 34pts (35%) ASCT as consolidation after thalidomide/bortezomib-based regimens (18pts) or standard chemotherapy (16pts) (ASCT group); 4pts received only standard chemotherapy and were excluded from analysis. Median follow-up from diagnosis was 56 months for both groups, range 13-148mo in TB and 17-118mo in ASCT. Baseline characteristics of the two groups, including age (median 62), were similar as well as the CR/VGPR and ORR rates obtained after first-line treatment (TB 43% and 87%; ASCT 53% and 79%, respectively). The subgroups also did not differ in median duration of first response and median time to second treatment, which were 17 and 26 months respectively, in both groups. Proportion of patients receiving a double ASCT was significantly higher in TB (47%) as compared to ASCT (9%) (P=0.03). *Results.* After second line therapy ORR (CR+VGPR+PR) was 85% in ASCT group, significantly better than TB group (49%) (P=0.0004). The second CR/VGPR rate was significantly higher after ASCT (41%) than after TB (20%) (P=0.033). Moreover, statistically significant differences in ORR were not observed in patients undergoing second-line consolidation ASCT, such as with thalidomide/bortezomib-based regimens (94%) or standard chemotherapy (75%) as reinduction (P=0.16). After a median follow-up from second-line treatment of 28 months (range 1-101mo), 2-yrs PFS was 24% after TB (median 18mo) and 36% after ASCT (median 19mo) (P=NS). 2-yrs OS was 68% (median 38mo) and 88% (median 47mo) after TB and ASCT, respectively (P=NS). Moreover, 2-yrs PFS and OS were not statistically significant different for those pts treated with new drugs as reinduction versus standard chemotherapy, in ASCT group (P=0.24). *Conclusions.* The use of ASCT as consolidation of second line treatment increased both ORR and CR/VGPR rates, independently from the type of the debulking treatment used (chemotherapy versus novel drugs), but did not significantly impact on PFS and OS when compared with second line new drugs only-based regimens. Novel drugs may be reserved for those patients not eligible for second line ASCT and preserved for effective third-line treatment in the other patients.

0379

SAFETY AND EFFICACY OF LENALIDOMIDE PLUS DEXAMETHASONE IN ELDERLY NEWLY DIAGNOSED MULTIPLE MYELOMA PATIENTS 70 YEARS OF AGE AND OLDER

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Background. In elderly patients with newly diagnosed multiple myeloma (MM), bortezomib-melphalan-prednisone (VMP) and melphalan-prednisone-thalidomide (MPT) are now considered the standards of care. Recently, the high efficacy and tolerability of lenalidomide plus dexamethasone (Len/dex) as initial therapy for MM was established in two randomized trials. Due to its safety profile and convenience, Len/dex is increasingly used as initial treatment in frail elderly patients, but data on the safety and efficacy in patients aged 70 and older are limited. *Aims.* To assess the efficacy and the toxicity of Len/dex combination as primary therapy in newly diagnosed elderly MM patients 70 years of age and older. *Methods.* 42 consecutive patients seen at Mayo Clinic between 2004 and 2008, aged >70 years old, who received induction treatment with Len/dex were studied in this retrospective study. Lenalidomide was given at a dose of 25 mg/day, days 1-21 on a 28-day cycle. All patients received dexamethasone, either at high-dose (n=16) (40 mg orally on days 1-4, 9-12, and 17-20) or at low-dose (n=26) (40 mg orally day 1, 8, 15, 22); each cycle was repeated every 4 weeks. Treatment was continued until progression, relapse or unacceptable toxicity, or could be stopped at the physician's discretion. Patients (n=11) were allowed to receive transplant if they wished and were deemed eligible. Outcome was analyzed on an intention-to-treat basis. Time-to-event analysis was performed using the Kaplan-Meier method. *Results.* On intention-to-treat analysis, nine of 42 (21%) patients achieved a complete response, 18 of 42 (42%) at least a very good partial response, and 34 of 42 (81%) at least a partial response. Median progression-free survival was 28 months, and the 3-year overall survival (OS) was 70%. When the analysis was restricted to patients who did not receive transplant, 3-year OS was 65%. Twenty-six (62%) of 42 patients experienced at least one grade 3 or higher extra-hematological adverse event: the most common toxicities were infections (8 patients), venous thromboembolism (7 patients) and fatigue (4 patients). Grade 3-4 hematological toxicities were reported in 11 patients, and were mainly represented by neutropenia. *Conclusions.* This cohort study shows the efficacy and tolerability of Len/dex in elderly patients with newly diagnosed MM 70 years of age and older. The 3-year OS results are comparable to those obtained with three-drug combination studies. Randomized phase III studies, comparing this non-alkylator containing regimen with alkylator-based combinations such as MPT or MPV are needed.

0380

LENALIDOMIDE INDUCES LONG-TERM RESPONSES IN PATIENTS WITH MULTIPLE MYELOMA RELAPSING AFTER MULTIPLE CHEMOTHERAPY LINES: IN PARTICULAR AFTER ALLOGENEIC TRANSPLANTATION

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Background. Lenalidomide is effective for the treatment of relapsed multiple myeloma (MM), but data on long-term responses are lacking. *Aims.* This retrospective multicenter study wanted to evaluate whether long-term responders exist and which patients' characteristics may predict the long-term response. *Methods.* Patients relapsing after >/=2 chemotherapy lines receiving lenalidomide were included. Response was defined as per International Uniform Response Criteria; long-term response was defined as >/=PR lasting >/=12 months, which is beyond the median PFS of registrative studies. OS and PFS were analyzed by Kaplan-Meier, relapse incidence (RI) by Cumulative Incidence method. Multivariate analysis was performed by logistic regression. 104 patients were enrolled in 13 Italian hematology centers: 61% had IgG, 27% IgA, 12% light chain MM. Median age was 60 yrs (range 35-84). Cytogenetic analysis at diagnosis was available in 56% of the patients, and 7% of them were considered at high-risk due to the presence of 17p- deletion. Median number of previous chemotherapy was 3 (range 2-7) and included autologous transplantation (autoSCT) in 64% and allografting (alloSCT) in 13% of patients. All patients received lenalidomide for 21 every 28 days per cycle at a daily dose of 25mg (84%) or at reduced dose due to hematologic toxicity (16%). Dexamethasone was administered with lenalidomide in 98% of patients, either at low (<=160mg/cycle, 86%) or at high-dose (12%). The median number of courses per patient was 6 (range 3-72). *Results.* Median follow-up was 375 days (84-2005). The responses were: 4% sCR, 8% CR, 10% VGPR, 52% PR, 15% SD and 11% PD. All the 13 alloSCT patients responded: 7 patients with follow-up >=12 months were long-term responders. One- and 2-year OS were 78% and 67%, PFS 52% and 37%, RI 48% and 63%. OS was reduced by high-risk cytogenetics (P=0.01) and high-dose dexamethasone (P=0.05). PFS was reduced by older (>60 years) age (P=0.01) and high-dose dexamethasone (P=0.03) whereas previous alloSCT improved PFS (P=0.05). RI was higher in patients with age >60 years (P=0.01), high-risk cytogenetics (P=0.05), and receiving high-dose dexamethasone (P=0.03). AlloSCT patients had a significantly lower RI (P=0.04). Forty-two of 104 (40%) patients were long-term responders to lenalidomide. Long-term responders had significantly better OS than no long-

term responders (P<0.001). Long-term responders did not significantly differ by age, isotype, previous autoSCT, dexametasone dose and chemotherapy lines. Long-term responders included 7 alloSCT patients compared to 0 alloSCT in no long-term responders group (P=0.01). Long-term responders had a better quality of response than no long-term responders (P<0.001): 45% PR or more versus 5%. In the multivariate regression model the quality of response was confirmed as the significant predictor of long-term response (P=0.04) and reduced relapse (P=0.01). *Conclusion*s. In conclusion, 40% of MM patients treated with lenalidomide and dexamethasone were long-term responders. All the allografted patients with follow-up >/=12 months were long-term responders, suggesting and immune-mediated activity of the drug. The quality of response was the best predictor of long-term response and better survival, indicating that also in the relapse setting the therapy should be aimed at the best tumor reduction.

0381

EFFECTIVENESS AND PATTERNS OF BORTEZOMIB USE IN A REAL-LIFE SETTING: THE VESUVE COHORT STUDY

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Background. Targeted therapies are a very important therapeutic progress in the treatment of multiple myeloma. Bortezomib, the first proteasome inhibitor, was registered in France in April 2004 as third line treatment and in April 2005 as second line treatment. To date, no evaluation of bortezomib in a real-life setting has been conducted in France *Aims.* The VESUVE cohort study was designed to describe patterns and evaluate effectiveness of bortezomib use in a real-life setting. *Methods.* VESUVE is a national multicentre cohort study, conducted in 60 French centres that included patients initiating bortezomib from May 2004 to April 2006, using nominative pharmacy dispensations and/or preparations. Among those identified, patients treated for multiple myeloma were followed for 36 months. For each patient, demographic data, clinical characteristics of multiple myeloma, and treatment data were collected from medical files using a standardized case report form. Response was assessed by an independent committee according to adapted International Myeloma Working Group criteria. The overall survival rate from the onset of bortezomib was assessed using the Kaplan-Meier method. *Results.* A total of 798 patients were followed: mean age was 65.6 years and 53.3% were men. Immunoglobulin myeloma was reported for 83.5% of patients (56.3% IgG, 25.1% IgA), light chain for 14.9% and non-secretory for 1.6%. More than one third of patients (34.1%) had a β2-microglobulin level higher than 3.5 mg/L. Cytogenetic exams were available for 340 patients. Among them, 35.0% had a deletion of chromosome 13. Bortezomib was administered as first line treatment in 0.5% of patients, as second line in 17.7%, as third line in 33.3%, as fourth line and more in 48.5%. The median number of bortezomib cycles was 4, and 6.3% of patients completed more than eight cycles. Baseline bortezomib dose was 1.3 mg/m² for 75.7% of patients, 42.9% received bortezomib alone, 43.0% in association with dexamethasone. Response was not assessed in 79 patients (single cycle) and in 126 (missing data). They were comparable for baseline prognostic factors with the 593 evaluated patients. For the latter, the overall best response rate was 58.4% (2.2% complete, 13.2% very good partial, 43.0% partial). Disease was stable for 35.4% of patients and progressive for 6.2%. The overall survival rate from the onset of bortezomib was 61.1% at 1 year [95%CI 57.6-64.4], 42.2% at 2 years [95%CI 38.7-45.7] and 31.1% at 3 years [95%CI 27.8-34.4]. The median overall survival was 19.2 months. *Conclusions*. This observational study shows that conditions of use in a real-life setting may differ from those of the summary of product characteristics in terms of concomitant treatment or number of cycles. Nevertheless, the effectiveness was close to the efficacy reported in clinical trials.

0382

THALIDOMIDE AND DEXAMETHASONE AS SALVAGE THERAPY AT FIRST RELAPSE IN PATIENTS WITH MULTIPLE MYELOMA: ANALYSIS OF LONG-TERM CLINICAL OUTCOMES

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Aim of the present analysis was to evaluate the long-term outcomes of a series of 100 patients who received thal-dex as salvage therapy at first relapse after prior ASCT or conventional chemotherapy. By study design, thal was started at the dose of 100 mg/daily for two weeks and then escalated to 200 mg/daily, provided that the initial tolerance was acceptable. Otherwise, thal was continued at the initial dose until progression. Dex was given at a monthly dose of 160 mg. The first 60 patients did not receive any thromboprophylaxis, while fixed low-dose warfarin (0.25 mg/day) was added to thal-dex in the subsequent 40 patients. Median age of the patients was 62 years. Median time from start of first-line therapy to thal-dex was 34 months. Up-front therapy for MM had included ASCT, either single (30%) or double (42%), while the remaining 28 patients had previously received conventional chemotherapy. 59% of the patients were treated with a fixed thal dose of 100 mg/daily. Overall, median duration of thal-dex therapy was 14 months. 65% of the patients stayed on treatment beyond the achievement of the best response or plateau phase; median duration of thal in these patients was 22 months (range 1-79). The most frequent adverse events were constipation (42%, grade III 8%), peripheral neuropathy (58%, grade III 5%), bradycardia (20%, grade III 0%) and skin rash (11%, grade III 1%). Venous thromboembolism was recorded in 7 patients (3 not receiving any thromboprophylaxis), at a median of 8 months (range 3-11) from the start of thal-dex therapy. The frequency of grade III neuropathy was significantly higher in patients receiving thal 200 mg/daily in comparison with those treated with 100 mg/daily (8.5% vs 1%, respectively, P=0.01). Discontinuation of thal due to toxicity was recorded in 8 patients after a median of 12 months. On an intention to treat basis, 46% of patients achieved at least a partial response at a median time of 3 months from the start of thal-dex treatment; the response rate was not significantly different between patients receiving thal 100 mg/daily and those treated with 200 mg/daily. The median duration of response (DOR) was 28 months, while the median time to next therapy was 15.5 months. With a median follow up of 25 months, median OS, TTP and PFS were 43, 22 and 21 months, respectively. TTP and PFS were significantly longer for patients responding to thal-dex therapy (TTP: 34 months vs 15 months for non-responders, P=0.005; PFS: 28 months vs 12 months for nonresponders, P=0.001, respectively). Median survival after relapse from thal-dex therapy was 26 months. In conclusion, low dose thal-dex was an effective treatment of first relapse in MM, yielding DOR, OS and EFS comparable to those reported with other novel agents when used in the same setting of patients. Low-dose thal-dex was generally well tolerated, as reflected by the long stay on treatment in the absence of progression (median: 25 months) and a low discontinuation rate (8%).

0383

POST-APPROVAL SAFETY STUDY (PASS) OF LENALIDOMIDE COMPARED WITH OTHER TREATMENTS IN PATIENTS WITH RELAPSED OR REFRACTORY MULTIPLE MYELOMA: FIRST REPORT ON 518 PATIENTS

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Aim and *Background.* Lenalidomide plus dexamethasone was EMEA approved 2007 for the treatment of multiple myeloma (MM) patients who have received at least one prior therapy. Subsequently, an observational patient cohort study has been implemented to characterize the safety profile of lenalidomide in a normal clinical practice and to place the incidence of adverse events (AE) into context with those occurring in second line or later MM patients receiving other treat-

ments. *Methods.* Data has been collected prospectively from 518 MM patients in 117 institutions in 14 European countries. Patients had received at least one prior therapy and were commencing a new treatment in accordance with normal clinical practice with prescribed medication. No additional treatments or investigations outside of normal clinical practice were required. Data cut for this analysis was December 18th, 2009. *Results.* Of the 518 patients, 321 received lenalidomide plus dexamethasone, 105 bortezomib, 36 thalidomide, 28 other therapies and 28 had missing data. The median age of the total group was 70 years (range, 38-92), 56.9% were male. Most patients (75.4%) had a good performance status (ECOG 0-1) but 24.6% had an ECOG status of 2-4. The median number of previous treatment lines was 2 (1-6), 50.9% had two previous lines and 30.9% three or more. There were no important demographic or clinical differences in the baseline characteristics between the patient cohorts. Table 1 shows the NCI grade 3-4, AEs in the respective cohorts. 24.9% of patients in the lenalidomide cohort discontinued therapy. Patients in the lenalidomide cohort had a median Kaplan-Meier estimated treatment duration of 6.4 months. Discontinuation percentages and estimated median treatment duration for the bortezomib and thalidomide cohorts were 46.7% (3.6 months) and 41.7% (5.8 months), respectively. Primary reasons for discontinuation were adverse events (lenalidomide, 6.2%; bortezomib, 13.3%; thalidomide, 11.1%) and progression of disease (lenalidomide, 7.5%; bortezomib, 11.4%; thalidomide, 13.9%). *Conclusions.* In unselected groups of patients from normal clinical practice treated with lenalidomide, bortezomib, thalidomide or other therapies, lenalidomide demonstrates an acceptable safety profile. There are no major differences in the incidence of adverse events between lenalidomide, bortezomib and thalidomide treated patients. The overall discontinuation rate among lenalidomide-treated patients was roughly half that of patients treated with bortezomib or thalidomide, despite a longer treatment duration. Discontinuation of treatment due to adverse events or disease progression occurred less frequently in the lenalidomide cohort than in the thalidomide or bortezomib cohorts.

Events (NCI grades) (At least one event occurred)	Lenalidomide (N = 321)	Bortezomib (N = 105)	Thalidomide (N = 36)	Other Treatment (N = 28)
Event leading to death	5.0%	7.6%	5.6%	0%
Any grade 3–4 event	30.5%	33.3%	36.1%	14.3%
Drug-related grade 3–4 event	19.6%	17.1%	16.7%	7.1%
Drug-related serious event	10.6%	11.4%	11.1%	10.7%

Table 1. AEs according to NCI (v. 3) grading.

0384

A PHASE IB DOSE-ESCALATION STUDY OF ORAL PANOBINOSTAT (LBH589) AND IV BORTEZOMIB IN PATIENTS WITH RELAPSED OR RELAPSED AND REFRACTORY MULTIPLE MYELOMA

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Background. Panobinostat (LBH589) is a potent pan-deacetylase inhibitor that has been shown *in vitro* to inhibit several HDAC isoenzymes including HDAC6, a key factor in both the formation of aggresomes and function of the Hsp90 chaperone protein. The combination of panobinostat and the proteasome inhibitor bortezomib has demonstrated synergistic cytotoxicity in multiple myeloma (MM) in both *in vitro* and *in vivo* studies which has been hypothesized to occur, in part, through inhibition of proteasome and aggresome function. *Aims.* The aim of this Phase IB dose-escalation study is to identify the maximum tolerated dose (MTD) of panobinostat and bortezomib when these are administered in combination in patients with relapsed or relapsed and refractory MM. *Methods.* Patients with relapsed or relapsed and refractory MM received panobinostat (p.o. thrice weekly) and bortezomib (i.v. Days 1, 4, 8, 11) on a 21-day cycle to establish the MTD of the combination. *Results.* As of Oct 9 2009, 38 patients were treated in 5 cohorts (see table). The median age of patients treated on the study was 62 years (range 46-78). Patients received a median of 2 prior therapies (range 1-7), including stem-cell transplant (n=30) and bortezomib (n=22; 13 bortezomib-refractory). No dose-limiting toxicities (DLTs) occurred in cohorts 1 and 3, 1 patient had DLT in cohort 2 (neutropenia), 4 had DLTs in cohort 4 (2 thrombocytopenia, 1 pneumonia/thrombocytopenia/neutropenia, 1 fatigue), and 1 had DLT in cohort 5 (thrombocytopenia/asthenia/dizziness). Hematologic adverse events (AEs): included Grade 3/4 thrombocytopenia (n=30), neutropenia (n=23), and anemia (n=6). Non-hematologic AEs included diarrhea (n=23), nausea (n=18), pyrexia (n=17), fatigue (n=16), and asthenia (n=13). There have been no treatment-related deaths. Responses (≥MR) were observed in 26/38 patients (68%) across all cohorts. Of note, responses were observed in 8/13 (62%) of bortezomib-refractory patients. Management of AEs, including thrombocytopenia, by dose reduction or interruption, allowed for longer treatment duration in cohort 3. Patients treated in cohort 3 also demonstrated the most favorable efficacy profile with 7/8 patients responding and no patients demonstrating progressive disease. Safety results supported the further enrolment of patients in a 6th cohort at the same dose level to confirm MTD. Altogether, these results led to the recommendation of the cohort 3 dose level for further evaluation in clinical trials, with a modified dose-schedule incorporating a week-long rest period. *Summary and Conclusions.* The combination of oral panobinostat and i.v. bortezomib has a predictable and manageable safety profile with promising activity in patients with relapsed or relapsed and refractory MM, including patients with bortezomib-refractory MM. Review of data of at least 12 evaluable patients treated at 20 mg panobinostat plus bortezomib 1.3 mg/m^2 is ongoing to confirm the MTD. This dose level, and modified schedule with panobinostat 2 weeks on and one week off will be used in phase II and III PANORAMA trials based on this combination in order to optimize therapy duration.

Cohort	**1**	**2**	**3**	**4**	**5**
Panobinostat dose	10 mg	20 mg	20 mg	30 mg	25 mg
Bortezomib dose	1.0 mg/m^2	1.0 mg/m^2	1.3 mg/m^2	1.3 mg/m^2	1.3 mg/m^2
No. of patients: Total (BTZ-refractory)	**7 (4)**	**7 (5)**	**8 (2)**	**7 (0)**	**9 (2)**
CR		1 (0)	2 (0)	1 (0)	
VGPR	1 (0)				1 (0)
PR		3 (3)	3 (1)	4 (0)	6 (2)
MR	1 (1)		2 (1)	1 (0)	
SD*			1 (0)	1 (0)	1 (0)
PD	4 (2)	3 (2)			
NE	1 (1)				1 (0)

*SD: not meeting criteria for CR, VGPR, PR, MR, or PD.

Table 1.

0385

A PHASE I STUDY OF VORINOSTAT, LENALIDOMIDE, AND DEXAMETHASONE FOR RELAPSED AND REFRACTORY MULTPLE MYELOMA

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Background. Multiple myeloma (MM) is the second most common hematologic malignancy. Although novel treatment combinations have improved outcomes, new drug combinations are needed for this otherwise incurable disease. Vorinostat, an oral histone deacetylase inhibitor approved in the United States for the treatment of advanced cutaneous T-cell lymphoma, alters gene expression and protein activity, promoting MM cell death through multiple pathways. In preclinical studies, vorinostat was shown to synergistically enhance the anti-MM activity

of bortezomib and immunomodulatory and antiangiogenic drugs, including lenalidomide, with or without dexamethasone. *Aims.* The primary objective of this phase I, multicenter, open-label, investigational study was to determine the maximum tolerated dose (MTD) of vorinostat plus lenalidomide and dexamethasone in patients with relapsed or relapsed and refractory MM. Secondary objectives included determination of overall safety, tolerability, response rate, duration of response, and time-to-progression (TTP). *Methods.* Patients were enrolled sequentially into 1 of 5 escalating doses of the combination regimen using a standard 3+3 design for ≤8 cycles. Patients who tolerated the regimen and had clinical benefit were eligible for enrollment in an extension phase. Toxicity was evaluated using the National Cancer Institute CTCAE v3.0. Response was assessed using modified European Group for Blood and Marrow Transplantation criteria and International Myeloma Working Group Uniform Criteria. Safety and efficacy data were assessed with summary statistics, except for TTP, which was estimated by Kaplan-Meier method. *Results.* As of January 22 2010, 31 patients have been treated and are evaluable for safety. Most patients had received prior bortezomib (65%), thalidomide (68%), or lenalidomide (42%), with a median of 3 prior therapies (range, 1-12). Adverse events (AEs) were generally mild or moderate in severity. Common ≥grade 3 drug-related AEs (all cycles) included neutropenia (23%), thrombocytopenia (16%), diarrhea (13%), and fatigue (10%). There were 10 serious drug-related AEs among 6 patients, and 7 discontinuations due to toxicity. One dose-limiting toxicity (grade 3 diarrhea lasting >48 h) was observed at the maximum assessed dose (level 5; Table), but a MTD was not reached. There were no treatment-related deaths. Of 30 patients evaluable for response, 26 (87%) experienced at least stable disease (SD). Best responses included 2 complete (CR), 2 very good partial (VGPR), 11 partial (PR), and 4 minimal response (MR), with 7 patients achieving SD and 4 developing progressive disease, resulting in an overall response rate (ORR) (≥MR) of 63% (95% CI 44%-80%). Of 13 evaluable patients who received prior lenalidomide, SD or better was observed in 9 (69%), with 1 VGPR, 3 PR, 1 MR, and 4 SD, resulting in an ORR (≥MR) of 38% (95% CI 14%-68%). Responses proved durable, with a median TTP of 33 weeks (95% CI, 24-57 weeks). *Conclusions.* Preliminary results from this study suggest that vorinostat plus lenalidomide and dexamethasone is a convenient, effective, and generally well-tolerated oral regimen for relapsed/refractory MM. An MTD was not reached. Notable activity was observed in patients who had received prior lenalidomide, bortezomib, or thalidomide. Evaluation of this regimen in a phase II study is planned.

	Dosing					
Dose Level	Vorinostat,* mg QD	Lenalomide,† mg QD	Dexamethasone,‡ mg QD	Evaluable for DLT,§ n	Maximum Cycles,¶ n	DLT,¶ n
1	300	10	40	3	19	0
2	400	10	40	3	15	0
3	400	15	40	3	11	0
4	400	20	40	3	14	0
5	400	25	40	6 (+10[#])	13	Diarrhea (grade 3), 1

DLT=dose-limiting toxicity; QD=once daily.
28-d cycles. Concomitant daily aspirin recommended.
*7 d on; 7 d off. Days 1–7 and 15–21.
†Days 1–21.
‡Days 1, 8, 15, and 22.
§8/31 patients (across all dose levels) remain ongoing as of January 22, 2010.
¶Maximum treatment cycle for which ≥1 patient received ≥1 dose.
#10-patient expansion cohort enrolled at dose level 5 with no formal DLT assessment.

Table.

0386

INTERIM ANALYSIS OF A PHASE III RANDOMIZED, DOUBLE-BLIND STUDY OF VORINOSTAT OR PLACEBO COMBINED WITH BORTEZOMIB IN RELAPSED MULTPLE MYELOMA

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Background. Although current treatments for multiple myeloma (MM) have significantly improved initial clinical response, patients inevitably relapse and/or become refractory to approved treatments. This urgent and unmet need has stimulated the development of new targeted approaches and treatment combinations. Vorinostat, approved in the United States for the treatment of advanced cutaneous T-cell lymphoma, is a histone deacetylase (HDAC) inhibitor that alters gene expression and protein activity. The preclinical synergistic effect of vorinostat with bortezomib to inhibit aggresome formation and induce MM cell apoptosis was confirmed in phase I trials and led to a large phase III program to evaluate the clinical benefit of this drug combination for the treatment of relapsed MM. *Aims.* Vantage 088 is an ongoing, global, Phase III, randomized, double-blind study to investigate the efficacy and tolerability of vorinostat vs placebo in combination with bortezomib in relapsed MM patients. *Methods.* Patients with relapsed MM and progressive disease after 1-3 prior anti-myeloma treatments are being enrolled in this investigational study. Patients who received prior bortezomib must have had ≥minimal response on therapy and not be bortezomib-refractory. Patients receive intravenous bortezomib 1.3 mg/m^2 on days 1, 4, 8, and 11 and oral vorinostat 400 mg (or matching placebo) once daily on days 1 to 14 of each 21-day cycle. The primary endpoint is progression-free survival, with a planned accrual of 742 patients. Secondary and exploratory endpoints include tolerability, overall survival, time to progression, objective response rate, and patient-reported outcomes (PROs). Efficacy is evaluated using European Blood and Marrow Transplantation Group criteria. Instruments for PROs, including quality of life (QoL) questionnaires for cancer patients (EORTC QLQ-C30) and multiple myeloma patients (EORTC QLQ-MY20), and the EuroQol EQ-5D, are unique aspects to the study design; the use of symptom scales, including disease symptoms and side effects of treatment, coupled with QoL assessments will provide opportunities to correlate PROs with efficacy and safety data. Safety is assessed according to NCI-CTCAE, version 3.0 and clinical and laboratory safety measurements are recorded. *Results.* As of February 2009, 196 patients were enrolled (range, 1-12 cycles). The median number of prior treatments was 1.8 (range, 1-3). Safety data were collected from all patients. As of August 2009, the independent data monitoring committee (DMC) had evaluated safety data from 39 patients. Most common adverse events (AEs) included gastrointestinal (51%), hematologic (28%), and general symptoms (eg, fatigue, [26%]). Most common grade ≥3 drug-related AEs were reported in 9 patients (thrombocytopenia [27%] platelet count decrease [17%], nausea [17%]). Only 2 grade 5 AEs occurred (sepsis, renal failure/plasmacytosis; neither was treatment-related). 4 patients (10%) discontinued (AE, death, consent withdrawn, progressive disease [n=1 patient each]). The DMC recommended continuation of the study without modifications based on available safety data. *Summary/Conclusions.* This is a large international randomized trial that has passed the initial safety evaluations by DMC and is accruing patients rapidly all over the world. Updated safety data and enrollment status from the second DMC meeting held in April 2010 will be presented.

0387

COMBINED VORINOSTAT AND BORTEZOMIB IN BORTEZOMIB-REFRACTORY PATIENTS WITH RELAPSED/REFRACTORY MULTIPLE MYELOMA: INTERIM ANALYSIS OF A PHASE IIB STUDY

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Background. Despite significant therapeutic advances in the treatment of multiple myeloma, a cure remains elusive and patients ultimately relapse and become refractory to available therapies. New treatments and novel treatment combinations are being assessed to address this unmet need. Vorinostat is a histone deacetylase (HDAC) inhibitor that alters gene expression and protein homeostasis. Approved in the United States for the treatment of advanced cutaneous T-cell lymphoma, vorinostat may target multiple pathways to increase myeloma cell apoptosis in combination with other small-molecule targeted therapies, such as bortezomib. In two phase I studies in relapsed/refractory (RR) MM patients, vorinostat in combination with bortezomib showed objective response rates (ORR; ≥partial response) of up to 42% in all patients, including bortezomib-refractory patients, and an overall clinical benefit up to 90%. *Aims.* Vantage 095 is an ongoing phase IIb open-label study to investigate the efficacy and tolerability of adding vorino-

stat to bortezomib in patients who are refractory to bortezomib and at least one immunomodulatory drug (IMiD) regimen and are ineligible for other approved regimens. *Methods.* Patients with RR MM who had ≥2 prior antimyeloma therapies were enrolled in this investigational study. All patients were refractory to bortezomib and relapsed, refractory, intolerant, or ineligible for other MM therapies, including IMiDs. Patients received intravenous bortezomib 1.3 mg/m^2 on days 1, 4, 8, and 11 and oral vorinostat 400 mg once daily on days 1 to 14 of each 21-day cycle. The primary endpoint is ORR with a planned accrual of 142 patients. Efficacy was evaluated using EBMT criteria. Safety was assessed according to NCI-CTCAE, version 3.0. Clinical and laboratory safety measurements were recorded. *Results.* As of February 2010, 85 patients were enrolled (range, 1-11 cycles). The median number of prior treatments was 6 (range, 2-17). Interim analysis for futility was triggered when the first 43 patients were enrolled and completed ≥3 cycles of therapy. Final adjudicated efficacy data for those 43 patients and the safety profile based on 58 patients treated at the time were reviewed by the independent data monitoring committee (DMC; data cut-off November 10, 2009). The ORR (CR+PR) was 16% (95% CI, 7-31%); minimal response and no change were observed in 5% and 51% of patients, respectively. Safety data were collected from all patients who received study drug (n=58) and were evaluated by the DMC. Fifty-three patients experienced adverse events (AEs) (most commonly including blood and lymphatic system, gastrointestinal, and general disorders). Drug-related grade ≥3 serious AEs were reported in 9 patients (most commonly thrombocytopenia [n=3], anemia [n=2], diarrhea [n=2]), one of which was grade 4 tumor lysis syndrome. No drug-related grade 5 AEs occurred. As of the data cut-off date, a total of 18 patients discontinued due to progressive disease and 7 owing to AEs. Based on passing the futility endpoint and the safety profile, the DMC recommended continuation of the study without modifications. *Summary/Conclusions.* This therapeutic regimen appears to have acceptable tolerability. Final data will confirm the potential of vorinostat combined with bortezomib in this advanced myeloma population.

0388

PX-171-006, A PHASE IB DOSE-ESCALATION STUDY OF CARFILZOMIB + LENALIDOMIDE + LOW-DOSE DEXAMETHASONE IN RELAPSED AND/OR REFRACTORY MULTIPLE MYELOMA

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Background. Carfilzomib (CFZ) is a specific, irreversible proteasome inhibitor with promising single-agent activity in relapsed/refractory multiple myeloma (R/R MM). In previous Phase II trials, CFZ demonstrated a favorable side-effect profile with minimal myelosuppression. Lenalidomide (LEN) + dexamethasone (Dex) is a standard of care for R/R MM. In preclinical studies, LEN sensitized MM to proteasome inhibition with bortezomib (BTZ), suggesting that combination therapy may enhance clinical activity. BTZ+ LEN/Dex, while active in R/R MM, has toxicity limitations. CFZ + LEN/Dex (CRd) may provide superior activity to LEN/Dex alone. *Aims.* This open-label, Phase Ib dose-escalation study evaluates the safety, activity, and maximum recommended dose (MRD) of CRd in R/R MM. *Methods.* CRd was evaluated at 6 dose levels (≥3 subjects each). An additional ~40 patients have been enrolled in an expansion cohort at the highest dose level tolerated or the cohort 6 dose. Eligible patients include those with R/R MM following 1-3 prior therapies (including prior LEN and/or BTZ). CRd was given on 28 day cycles (C) in 6 cohorts. Primary endpoints include safety and establishment of the MRD. Additional endpoints include overall response rate (ORR) (≥ PR) as assessed by IMWG Criteria, with secondary assessment of clinical benefit response (CBR: ≥ minimal response [MR]) using EBMT Criteria. *Results.* To date, 40 patients have been enrolled in cohorts 1-6 and 40 in the cohort 6 expansion. 27/32 patients in cohorts 1-5 are evaluable for safety and 29/32 for response. Patients were heavily pre-treated; 72% received prior BTZ and 87.5% received prior LEN or thalidomide (Thal). 47% of patients were refractory to their last therapy; >84% of patients had a history of neuropathy with 67% BTZ- or Thal-related. No treatment emergent fatigue, neuropathy, or thrombotic events ≥ Grade (G) 3 were observed. Hematological AEs ≥G3 (thrombocytopenia [n=6], anemia [n=4], and neutropenia [n=6]) were reversible. 4 patients had drug-related SAEs as follows: transient G3 sinus bradycardia, G3 upper respiratory tract infection, febrile neutropenia, and G3 diarrhea + G3 urinary infection. ORR and CBR for the 29 evaluable patients are 59% and 72%, respectively. Response data is shown in the table. Initial responses improved with continued therapy, (up to 18 C). Median duration of response has not been reached (median follow-up 5.2 months). No dose-limiting toxicities or deaths attributed to study treatment have been observed. Updated data will be presented (Table). *Conclusions.* CFZ (20/27 mg/m^2) in combination with full dose LEN (25 mg) and Dex (40 mg Qwk) (CRd) is well-tolerated in patients with R/R MM, even in those with a history of neuropathy. Non-overlapping toxicity profiles of CFZ and LEN permits combination at full doses for extended durations up to 18 C. Prior therapy with BTZ or LEN and/or Thal, or disease refractory to the immediately preceding regimen did not preclude achieving CR, VGPR, PR, or MR with CRd. Based on these data, a Phase III international trial of CRd vs. Rd in relapsed MM will commence in 2010.

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	CRd: Cohorts 1–5 (CFZ: 15 to 20 mg/m2; LEN: 10 to 25 mg)		
Response	**Relapsed (n=16)**	**Refractory (n=13)**	**Overall (n=29)**
≥ CR/nCR	5 (31)	1 (8)	6 (21)
≥ VGPR	7 (44)	4 (31)	11 (38)
≥ PR	9 (56)	8 (62)	17 (59)
≥ MR	11 (67)	10 (77)	21 (72)

Table.

0389

ELOTUZUMAB IN COMBINATION WITH LENALIDOMIDE AND LOW DOSE DEXAMETHASONE IN PATIENTS WITH RELAPSED OR REFRACTORY MULTIPLE MYELOMA: A PHASE 1/2 STUDY

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Background. Elotuzumab is a humanized antibody against CS1, a cell surface glycoprotein that is highly expressed in multiple myeloma (MM). Elotuzumab induces antibody-dependant cytotoxicity (ADCC) against primary MM cells in the presence of peripheral lymphocytes (PBMC). The elotuzumab-mediated ADCC is significantly enhanced when PBMC are pretreated with lenalidomide (len). In mouse xenograft MM models, elotuzumab given in combination with len demonstrated significantly enhanced antitumor activity compared to len alone. *Aims.* The study objectives evaluated the maximum tolerated dose (MTD), safety, pharmacokinetics (PK), and efficacy of elotuzumab in combination with len and low dose dexamethasone (dex). *Methods.* Elotuzumab (IV infusion) in 3 escalating dose cohorts (5, 10, and 20 mg/kg) was given weekly for the first 2 cycles and then every other week of subsequent cycles. Len (25 mg PO) was given on days 1 to 21 of each 28-day cycle. Dex was given weekly at 40 mg PO. The first 5 patients (pts) were treated for 6 months before a protocol amendment to continue treatment until progression was approved. *Results.* A total of 29 pts with a median age of 60 years were enrolled; 28 pts received elotuzumab. One pt was withdrawn from the study prior to receiving study drug due to investigator's decision. Pts had a median of 3 prior MM treatments including thalidomide (59%), bortezomib (69%), or len (21%) with 41% of pts refractory to their last MM therapy. Three pts each received 5 mg/kg and 10 mg/kg and 22 pts received 20 mg/kg of elotuzumab. No dose-limiting toxicity (DLT) was observed during dose-escalation and a MTD was not established. Two pts in the 20 mg/kg cohort discontinued due to infusion reactions, 1 grade (G) 4 hypersensitivity (related to elotuzumab) and G3 stridor (related to elotuzumab/dex). Additional unrelated SAEs (1 each) included G2 atrial fibrillation and enteritis, G3 chest pain, pneumonia, sepsis, gastrointestinal haemorrhage, febrile neutropenia, diarrhoea, atrial fibrillation, acute renal failure, and metabolic acidosis, G4 diverticular rupture, and sepsis. Common adverse

events included fatigue, diarrhoea, constipation, anaemia, neutropenia, nausea, muscle spasms, pyrexia, asthenia, and dyspnoea. The ORR by IMWG criteria in 28 treated pts was 82% (64% PR; 18% VGPR). Pts naive to len (22 pts) demonstrated ORR of 95% (73% PR; 23% VGPR). Median time-to-progression (TTP) following a median of 4.5 cycles has not yet been reached. Preliminary PK analysis suggests trough serum antibody concentrations of elotuzumab at 10 mg/kg and 20 mg/kg doses are above target levels predicted based from preclinical models. Elotuzumab dosing also resulted in complete saturation of target sites on bone marrow plasma cells. *Summary/Conclusions.* The combination of elotuzumab with len/dex has a manageable safety profile and the ORR of 82%, including 95% in len-naive patients is encouraging. A 60 pt, randomized, phase 2 expansion has been initiated to further examine the efficacy and identify the optimal dose of elotuzumab. Updated safety and efficacy data including TTP will be presented at the meeting.

0390

A PHASE IB STUDY EVALUATING THE COMBINATION OF ORAL PANOBINOSTAT (LBH589) WITH LENALIDOMIDE AND DEXAMETHASONE IN PATIENTS WITH RELAPSED OR RELAPSED AND REFRACTORY MULTIPLE MYELOMA

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Background. Panobinostat (LBH589) is an orally bioavailable pan-deacetylase inhibitor targeting histone-mediated epigenetic and non-histone oncogenic pathways. The combination of panobinostat with the IMiD lenalidomide, and dexamethasone, demonstrated synergistic anti-multiple myeloma (MM) activity in preclinical models. *Aims.* This Phase IB, dose-escalation study sought to establish the maximum tolerated dose (MTD) of panobinostat in combination with lenalidomide and dexamethasone for the treatment of patients with relapsed or relapsed and refractory MM. *Methods.* Cohorts of ≥6 patients received panobinostat at one of four dose levels guided by Bayesian design (5 mg, 10 mg, 20 mg, or 25 mg; po thrice weekly every week) in combination with a single dose of lenalidomide (25 mg po days 1-21) and of dexamethasone (40 mg po days 1-4, 9-12, 17-20, cycles 1-4; days 1-4, cycle 5 onwards), in a 28-day cycle. Dose limiting toxicities (DLTs), safety, tolerability, PK/PD, and preliminary efficacy were evaluated. *Results.* As of Dec 8, 2009, 46 patients had been treated at 4 panobinostat dose levels (cohorts 1-4): 5 mg (n=8), 10 mg (n=8), 20 mg (n=21), or 25 mg (n=9). Patients received a median of 2 prior therapies (range 1-8), including bortezomib (n=28), thalidomide (n= 29), lenalidomide (n=8), and/or stem cell transplant (n=36). Over half of patients (n=25) had refractory disease. DLTs were observed in 9/36 evaluable patients (cohorts 1-4, including 3/7 patients at 25 mg panobinostat): hematologic (n=4) (thrombocytopenia, neutropenia), QTcF prolongation (n=2), atrial fibrillation (n=1), fatigue (n=1), and pneumonia (n=1). In 46 patients evaluable for safety, common adverse events (AEs) (≥20% patients) included: hematologic (Grade 3/4 thrombocytopenia [41%] or neutropenia [37%]), and gastrointestinal, or muscular. Other Grade 3/4 AEs were: fatigue/asthenia, reduced appetite, insomnia, muscle weakness/metabolic (dexamethasone-related), respiratory infection, diarrhea, and dysgeusia. Two of 7 deaths on study (respiratory insufficiency secondary to infection and febrile neutropenia with respiratory infection and sepsis) were suspected as treatment-related and occurred at the 20 mg dose. One patient had DVT. Dose adjustment frequency was comparable in lower-dose cohorts, primarily due to dexamethasone-related AEs, but increased in the 25 mg cohort (≥1 reduction in 6 patients). The MTD confirmed by the logistic Bayesian model (based over cycle 1 DLTs) was panobinostat 20 mg dose level which was expanded to include ≥12 evaluable patients. Review of tolerability over longer-term therapy is ongoing and dose recommendation for further clinical evaluation will be reviewed. Disease assessment was available for 30 patients, with responses observed in 17 (57%): 1 sCR, 1 CR, 7 VGPR, and 8 PR. Seven (23%) patients had SD, and 6 (20%) patients had PD. Of 12 patients refractory to a prior IMiD line and currently evaluable for response, 5 responded to study therapy. *Summary and Conclusions.* In this first trial evaluating the combination of oral panobinostat with lenalidomide and dexamethasone in patients with advanced MM, preliminary efficacy was very encouraging with the safety data generally consistent with previously established profile. MTD in this combination was confirmed to be 20 mg of panobinostat at this dosing regimen and schedule.

0391

A MYC ACTIVATION SIGNATURE IS COMMON IN MM AND ASSOCIATED WITH SHORTER SURVIVAL THAT CAN BE OVERCOME BY BORTEZOMIB

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Background. We previously identified a MYC activation signature that suggests MYC activation may be an important event in the transition from monoclonal gammopathy of undetermined significance to multiple myeloma (MM). *Aims.* Study the clinical association and relevance of MYC activation in MM. *Methods.* We examined a Mayo Clinic MM cohort (n=101) treated with chemotherapy with or without stem cell transplantation that had gene expression done on Affymetrix U133A chips. We also analyzed a cohort of relapsed MM (APEX cohort) entered into a clinical trial comparing bortezomib (n=80) and dexamethasone (n=76) that had gene expression done on Affymetrix U1133A and B chip. The data was MAS5 normalized, log2 transformed and median centered for analysis. A MYC activation index (MAI) based on the median expression of genes constituting the MYC activation signature was generated. Genetic information included FISH detection of hyperdiploidy, t(11;14), t(4;14), 13 deletion, 17p13 deletion was available in the Mayo dataset. Clinical parameters including ISS stage, beta-2 microglobulin and plasma cell labeling index (PCLI) were also assessed. Association was assessed using Chi-square tests for categorical values. Comparison of continuous variables was using Mann-Whitney U Test. Survival curves were constructed using the Kaplan-Meier method and compared using log-rank test. In the Mayo cohort, overall survival (OS) was calculated from diagnosis to death. In the Apex cohort, progression free survival (PFS) and OS was calculated from trial entry to disease progression and death respectively. All patients consented to the study that was approved by the Institution Review Board of the Mayo Clinic. *Results.* In the Mayo cohort 68% had a high MAI (>1 arbitrary unit). A high MAI was associated with hyperdiploid (81% versus 62%, P=0.04), higher PCLI (median 1 versus 0.25, P=0.007), and ISS III (90% ISS III have high MAI versus 62% ISSI/II have high MAI, P=0.03). Patient with high MAI had significantly shorter survival (33 months versus 63.4 months, P=0.001). In the APEX cohort, a high MAI was associated with shorter survival compared to low MAI (16 months versus 31.9 months, P=0.01). However subgroup analysis according to treatment received showed that the prognostic impact of a high MAI is only present in patients treated with dexamethasone (9.6 months versus 36.9 months, P=0.002) but not with bortezomib (21.6 months versus 23.1 months, 0.6). We next assessed the impact of types of drug treatment within the 2 MAI categories. Response (57% versus 27%, P=0.03), PFS (6.3 months versus 2.9 months, P=0.02) and OS (21.6 months versus 9.6 months, log-rank P=0.04) was better for patients treated with bortezomib than dexamethasone in those with high MAI (37 treated with bortezomib, 28 treated with dexamethasone). However, PFS (5.6 months versus 3.4 months, P=0.34) and OS (31.9 months versus 23.1 months, P=0.4) were not different between bortezomib and dexamethasone treatment in those with low MAI (43 treated with bortezomib and 48 treated with dexamethasone). *Summary/conclusions.* MYC activation is more common in hyperdiploid MM and associated with shorter survival. This survival disadvantage seems to be overcome by bortezomib due to better response, PFS and OS.

0392

PHASE II STUDY OF CARFILZOMIB IN PATIENTS WITH RELAPSED OR REFRACTORY MULTIPLE MYELOMA AND VARYING DEGREES OF RENAL INSUFFICIENCY

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Background. Approximately 20% of patients with newly diagnosed multiple myeloma (MM) have concomitant renal impairment (RI) and >50% will experience it during the course of their disease. RI confers shorter survival due to higher tumor burden and prevents adequate dosing of active drugs by exacerbating side effects due to reduced renal clearance. Carfilzomib (CFZ) is a novel, highly selective peptide epoxyketone proteasome inhibitor with single agent activity in relapsed and/or refractory (R/R) MM. *Aims.* In previous Phase II studies, CFZ demonstrated activity in heavily pretreated patients with R/R MM including those with moderate RI. The present study was designed to evaluate the tolerability and PK properties of CFZ in R/R MM patients with RI, including those on dialysis. *Methods.* This open-label, multicenter phase II trial enrolled R/R MM patients with varying degrees of RI who had relapsed after ≥2 prior therapies. Patients were stratified based on their degree of RI (Table).

Evaluable Patients					
Cohort	**CrCL, mL/min**	**Enrolled**	**Efficacy**	**Safety**	**PK**
Normal	80	10	10	2	4
Mild RI	50-79	9	8	8	6
Moderate RI	30-49	9	7	6	6
Severe RI	<30	9	8	4	6
On hemodialysis		2	2	2	2
Total		**39**	**22**	**35**	**24**

AE	All G (n)	G 3/4 (n)
Fatigue	14	3
Anemia	10	9
Diarrhea	9	
Nausea	8	
Constipation	7	
Thrombocytopenia	7	6
Hypokalemia	7	
Creatinine increase		4
Mental status change		3

Table 1.

Study objectives included safety, PK, pharmacodynamics (proteasome inhibition in blood and PBMC) and efficacy (overall response rate [≥PR], clinical benefit response [≥MR; CBR], stable disease [SD] ≥6 wk, duration of response, and time to progression). Patients received CFZ 15 mg/m^2 IV on days 1, 2, 8, 9, 15, and 16 every 28 days for cycle 1, with escalation to 20 mg/m^2 in cycle 2 and 27 mg/m^2 in cycle 3 and thereafter, if tolerated. After response assessment, patients failing to achieve ≥PR by cycle 2 or CR by cycle 4 could receive "low dose"dexamethasone (40 mg/week). *Results.* To date, 39 patients have been enrolled. Patients received a median of 5 prior therapies (range 2-10). All had refractory MM and 42% had progressed during their last therapy. 97% had received bortezomib; all had received ≥1 immunomodulatory agent (87% thalidomide and 95% lenalidomide); 67% had prior stem cell transplant. Thus far, 22 patients received a median of 3 C (range 1-10+); 9 have completed ≥6 C. Side effects (AEs), including Grade (G) 3/4 AEs, are presented in the table. There were no appreciable differences among the 5 cohorts in either frequency or grade of CTC-AE. No QT/QTc prolongations were seen. PK/PD were similar across all cohorts; CFZ was undetectable in plasma within 3 h (t½ = 30-60 min) and did not accumulate after 2 C. Mental status changes were all reversible; two of the 3 Grade 3/4 change in mental status were unrelated to drug and 1 resolved with drug continuation. Proteasome inhibition 1 h post-dose ranged from 75-89% at doses of 15-20 mg/m^2. The CBR was 37% (8 PRs, 5 MRs) with an additional 37% SD (n=13). Two bortezomib-refractory patients achieved PR. Seven of 18 patients received dexamethasone with 2 exhibiting improved responses (1 PR and 1 MR). *Conclusions.* Patients with MM and substantial RI can receive CFZ without dose adjustment. Toxicities are mild and manageable with no apparent effect of baseline RI on AEs. Importantly, exacerbation of pre-existing peripheral neuropathy, RI, and myelosuppression were not observed. Responses are encouraging and further evaluation of CFZ in RI patients is ongoing.

0393

CARFILZOMIB, A NOVEL PROTEASOME INHIBITOR, EXHIBITS MINIMAL NEUROTOXIC AND PERIPHERAL NEUROPATHIC EFFECTS IN PRECLINICAL AND CLINICAL STUDIES

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Background. Treatment-induced peripheral neuropathy (TIPN) can be a debilitating and therapy-limiting complication in multiple myeloma (MM). Thalidomide and bortezomib (BTZ) are two therapies frequently associated with TIPN in MM. Carfilzomib (CFZ) a novel, selective proteasome inhibitor differs from BTZ both structurally and mechanistically. Preclinically, CFZ is not associated with the off-target activities seen with BTZ (Arastu-Kapur et al, Haematologica, 2009), is active in BTZ-resistant tumor cells (Kuhn et al, Blood 2007 and Suzuki et al, Blood 2009), and is not associated with neurotoxicity in chronic animal toxicity studies. (Kirk et al, Blood 2008). *Aims.* Single-agent CFZ has demonstrated activity in relapsed or refractory (R/R) MM and does not produce dose-limiting PN. Here we compare the effects of CFZ and BTZ in an *in vitro* neurotoxicity model and report on clinical experience with CFZ from two ongoing Phase II trials. *Methods.* Proteasome inhibitor effects were measured by high content analysis of fluorescent images for cell survival (Hoechst nuclear counterstain) and neurite degeneration (FITC-mouse anti-beta-III-tubulin) in an established neurotoxicity model using differentiated SH-SY5Y neuroblastoma cells. Changes in cell morphology and cell death were captured using phase contrast imaging and both cell viability and proteasome inhibition were measured. Serine proteases with a P1 selectivity of Leu/Phe/Tyr were identified from the MEROPS (peptidase) database as potential non-proteasomal targets of CFZ and BTZ activity. *Methods.* Data from two ongoing Ph II trials of single-agent CFZ in patients with R/R MM were pooled and included past history of neuropathy, neuropathic symptoms at baseline, findings on neurological exam, and FACT-GOG/NTx scores. Adverse event (AE) data were also collected. *Results.* SH-SY5Y cells showed a 40% reduction in neurite length/cell after 24 hrs of exposure to 10 nM BTZ, compared to both CFZ- or vehicle-treated cells. However, the level of proteasomal inhibition was equivalent in CFZ and BTZ treated cells. HtrA2/Omi, a mitochondrial serine protease with a role in neuron survival was identified in the MEROPS database. HTrA2/Omi was confirmed as a non-proteasomal target of BTZ (IC_{50} = 3 nM), but not CFZ (>10 μM). *Results.* At screening, 72% of 135 patients had symptoms of PN; 81% had a history of PN attributed to their prior MM therapy. Patients received a mean of 30.4 doses (range 2-72) of CFZ and 17% were treated for over 10 mo. 10% of patients had drug-related PN (primarily Grades 1/2). Grade 3 PN was reported in only 3 (2.2%) pts; there were no reports of Grade 4 PN. No missed doses or treatment discontinuations occurred due to PN. Only 1 (<0.01%) patient was dose-reduced due to neuropathy. *Conclusions.* Contrary to BTZ, CFZ-related PN is infrequent and not a dose-limiting toxicity. *In*

vitro, BTZ potently inhibits a neuronal cell survival factor, HtrA2, and demonstrates significantly more neurotoxicity compared to CFZ despite equivalent proteasomal activity. Clinically, PN is not a treatment-limiting toxicity of CFZ. These *in vitro* and clinical data indicate that neurotoxicity occurs via a proteasome-independent mechanism and severe PN is not a class effect of proteasome inhibitors.

0394

MLN4924, A NOVEL NAE INHIBITOR IN PATIENTS WITH MULTIPLE MYELOMA (MM) AND NON-HODGKIN'S LYMPHOMA (NHL): PHASE 1 DOSE-ESCALATION STUDY

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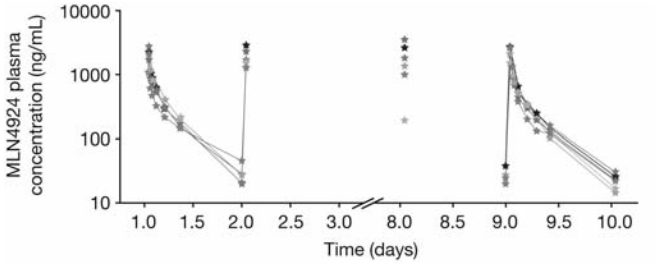
Background. MLN4924 is a first-in-class small-molecule inhibitor of NEDD8-activating enzyme (NAE). Inhibition of NAE prevents conjugation of NEDD8 to the Cullin Ring Ligases (CRLs), which inhibits proteasomal degradation of CRL substrates including proteins involved in cell cycle regulation, signal transduction (pIκBα), DNA replication (Cdt-1), and stress response (Nrf-2). *Aims.* The primary objectives of this open-label, phase-1, dose-escalation study were to determine the maximum tolerated dose (MTD) and tolerability of MLN4924, pharmacokinetics and pharmacodynamics in blood, and pharmacodynamic effects in skin. *Methods.* Patients ≥18 years with relapsed and/or refractory MM, any B or T-cell NHL, Hodgkin's lymphoma or Waldenström's macroglobulinemia after ≥2 prior lines of therapy were eligible. Escalating doses of intravenous MLN4924 (25, 50, 65, 83, 110, 147mg/m^2) were administered on days 1, 2, 8, and 9 of 21-day cycles (maximum 12 months' treatment). MTD was defined as the dose level closest to that predicted to result in dose-limiting toxicity (DLT) rate of 25%. For the pharmacodynamic analysis, peripheral blood mononuclear cells (PBMCs) and whole blood were isolated at screening, baseline, and following MLN4924 administration; skin biopsies for Cdt-1 and Nrf-2 assays were performed at baseline and after the second dose. RT-PCR was used to analyze expression of NAE-regulated genes in whole blood. *Results.* By data cut-off, 24 patients had been enrolled; median age 64 years, 63% male. Fourteen patients had MM (IgA n=4; IgG n=9; other n=1) and 10 had NHL (FL n=4; DLBCL n=2; MCL n=1; CLL/SLL n=2; lymphoplasmacytic lymphoma n=1). In MM patients, 11 had received prior autologous stem-cell transplant; all 14 patients had received previous bortezomib, 10 received prior thalidomide and 10 received prior lenalidomide.

Figure. Representative plasma PK profiles (110 mg/m^2).



Seven NHL patients received prior transplant and 10 received prior rituximab. Of the 20 DLT-evaluable patients, 4 experienced DLT: G4 febrile neutropenia (65 mg/m^2); G3 aspartate aminotransferase (AST) elevation (110 mg/m^2); G4 muscle cramps, and G2 myalgia that was considered dose limiting (both 147 mg/m^2). The MTD for this schedule was therefore determined as 110 mg/m^2. Patients received a median of 3 treatment cycles (MM=4 cycles; NHL=2). No treatment-related deaths were reported; one MM patient died due to progressive disease. Overall G≥3 AEs occurred in 54% of patients (all doses) and the most common included anemia, fatigue, increased AST, and hypophosphatemia. MLN4924 plasma concentrations declined in a multi-phasic manner after the end of infusion with relatively low pharmacokinetic variability. Pharmacokinetic profiles were similar following dosing on days 1 and 9, suggesting no readily apparent accumulation (Figure). The area under the concentration-time curve appears to increase linerarly with increasing mg/m^2. In PBMCs, NEDD8-Cullin was decreased and pIκBα expression was increased versus baseline, indicative of NAE inhibition. In whole blood, Nrf-2-regulated gene transcripts including NQO1 were increased after dosing compared to baseline. Cdt-1/Nrf-2 in skin increased above baseline following the second MLN4924 dose. Summary: MLN4924 inhibits NAE activity in blood and skin, indicating that it exerts predicted pharmacodynamic effects. Continued investigation of MLN4924 110mg/m^2 to more fully characterize safety, pharmacokinetics, and pharmacodynamics in MM and NHL is underway; two additional schedules are being investigated in subsequent patients.

Myeloproliferative Disorders -Biology 1

0395

JAK2V617F MUTATION PERSISTS IN BLASTS AND MATURE CELLS OF TRANSFORMED- JAK2V617F-POSITIVE-MYELOPROLIFERATIVE NEOPLASIA: A EUROPEAN LEUKEMIA NET (ENL) STUDY

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Background. Transformation to acute myeloid leukemia (AML) is a known complication of MPN but the role of JAK2V617F mutation is still undefined. In 2006, Campbell described a possible model for the development of a JAK2WT-AML in a patient with JAK2V617F-MPN and more recently, Theocharides et al. reported that in up to 53% of the patients who developed secondary AML from a JAK2-mutated MPN the mutation was no longer detectable; however the results obtained with DNA extracted from cells scraped or laser-capture-microdissected from bone marrow (BM) or peripheral blood (PB) smears, were confirmed in fresh samples only in few cases. *Aims and Methods.* In this study, we collected, by cell sorting, blast cells and mature myeloid cells (granulocytes, GRA) from whole BM aspirates of 34 newly diagnosed patients with AML secondary to MPN (18 derived from PMF; 9 from PV and 7 from ET) and analyzed the JAK2 status before and after leukemic transformation in selected cell compartments. To evaluate the modification in the JAK2 status before and during leukemic transformation we performed ASO-PCR and (QRT)-PCR assay on total BM of the MPN phase and sorted cell populations from AML phase. *Results.* At the time of MPN diagnosis, JAK2V617F was detectable in 22 of 34 patients (65%) (10 of 18 PMF; 9 of 9 PV and 3 of 7 ET). No cytogenetic abnormalities or MPL and JAK2-exone 12 mutations were detected at this stage. Median time to AML progression (TTP) was 5.09 years (yrs) (range 0.38 - 27.81). A significant difference (P=0.02) in TTP was found grouping patients according to JAK2 status during the MPN phase [JAK2WT-MPN n=12, TTP median 15.10 yrs (0.38-16.32); JAK2 mutated-MPN n=22, TTP median 4.07 yrs (0.67-27.81)]. Eight patients showed additional abnormalities involving chromosomes 1, 5, 7, 8, 9, 12, 14, 17 and 20 while no other AML-associated mutations (FLT3, NPM, CEBPA, RUNX1) were detectable at this stage. In our cohort of patients we found that JAK2V617F mutation was still present at the blast transformation in both compartments: CD34+ cells (blasts) and CD15+ cells (GRA) in 20 of 22 JAK2 mutated MPN (91%). Two of 22 patients (9%) developed JAK2V617F negative AML starting from a mutated PV with a mean TTP of 5.14 yrs. Interestingly, the WT status was confirmed in blast cells but also in GRA. Surprisingly we found a case of JAK2V617F mutated AML transforming from a WT-PMF. Also in this case the JAK2V617F positivity in the AML phase occurred in both GRA and blast compartments. No differences (P= 0.3) in the allele burden were found comparing MNCs from chronic phase with MNCs of leukemic transformations or comparing GRA with blasts in AML phase. *Conclusions.* In conclusion, these results contrast with the previous study in which the JAK2 mutation was lost in 53% of blasts during leukemia transformation. In our work, the loss of JAK2V617F mutation during AML progression is a rare event (9%). Additional studies in larger patient series and multivariate analysis are needed before a prognostic role of JAK2V617F mutation regarding time to leukemia transformation can be definitely assessed.

0396

PROMOTER DNA HYPERMETHYLATION DISTINGUISHES VARIANTS OF JUVENILE MYELOMONOCYTIC LEUKEMIA WITH DIFFERENT PRESENTATION AND PROGNOSIS

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Promoter DNA hypermethylation contributes to the malignant phenotype in myeloproliferative neoplasms and myeloid leukemia. It is barely known whether aberrant DNA methylation also occurs in juvenile myelomonocytic leukemia (JMML), and if so, whether it is associated with clinical presentation, hematology or prognosis of the disease. We used denaturing liquid chromatography to analyze peripheral blood or bone marrow samples from 111 children with JMML and 17 healthy controls for aberrant DNA methylation at 14 candidate gene loci. All children had been enrolled in the European Working Group of MDS in Childhood (EWOG-MDS) studies 98 or 2006 with informed consent from parents or guardians. The analyses identified 4 genes with promoter DNA hypermethylation in JMML: bone morphogenetic protein 4 (BMP4) (37/111 cases), calcitonin A (*CALCA*) (38/111 cases), cyclin-dependent kinase inhibitor 2B (*CDKN2B*) (25/111 cases) and retinoic acid receptor beta (*RARB*) (18/111). The 17 controls were unmethylated at these 4 loci. We noticed that methylation in JMML did not occur with even or random distribution across samples and genes. Instead, hypermethylation was detectable at all four loci in some samples but was limited to fewer genes in others. We therefore categorized the cohort into three groups: no methylation (51/111 samples), limited methylation (1 or 2 genes; 44/111 samples) or extensive methylation (3 or 4 genes; 16/111 samples). A correlative analysis of methylation groups with clinical or hematologic features showed that extensive methylation was strongly associated with older age and elevated percentage of hemoglobin F (HbF) at diagnosis (P<0.001). A weaker correlation was seen with genetic JMML subtype (mutant *PTPN11, KRAS/NRAS, NF1,* or *CBL*) (P<0.05), with *CBL* cases largely being unmethylated. By contrast, there was no significant association between methylation and leukocyte count, absolute monocyte count, platelet count, blast percentage in blood or bone marrow or spleen size. Importantly, the presence of hypermethylation at diagnosis predicted cases with poor outcome; the 5-year overall survival (OS) in the no methylation group was 0.68 [0.53-0.82], as compared to 0.31 [0.06-0.55] in the extensive methylation group (P<0.001). Among patients receiving hematopoietic stem cell transplantation, hypermethylation characterized cases with significantly increased probability of relapse: the 5-year relapse incidence in the no versus high methylation groups was 0.17 [0.08-0.34] versus 0.57 [0.36-0.90] (P<0.001). Lineage-specific cell sorting demonstrated that aberrant methylation was restricted to clonal cell populations and could be traced back to the $CD34^+$ JMML progenitor cell compartment. This observation supports the concept that DNA methylation is associated with early pathogenetic events in JMML. Furthermore, longitudinal analyses indicated that some cases had acquired a more extensively methylated phenotype at relapse. In summary, we observed that a high-methylation phenotype characterizes an aggressive biologic variant of JMML and suggest that DNA methylation is an important molecular predictor of outcome.

0397

COMPETITIVE POTENTIAL OF JAK2-V617F STEM CELLS

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Background. The myeloproliferative neoplasms (MPN), polycythemia vera (PV), essential thombocythemia (ET), and primary myelofibrosis (PMF), are clonal diseases caused by somatic mutations that are thought to occur at level of the hematopoietic stem cell. Based on data from mouse models, the JAK2-V617F mutation appears to be sufficient to cause MPN as a single step event, but in patients several lines of evidence indicate that additional mutations can precede the acquisition on JAK2-V617F. *Aims.* We developed a competitive repopulation assay

that allows us to assess the MPN-initiating potential of the stem cells that carry JAK2-V617F as a sole genetic alteration and clarify their proliferative and competitive abilities. *Methods.* We previously generated a Cre-LoxP inducible transgenic mouse model (FF1 mice) for the expression of human JAK-V617F. These FF1 mice have been crossed with the MxCre or SclCre mice and upon induction, the double transgenic mice primarily display a PV phenotype. Lethally irradiated wild type recipients were transplanted with a 1:1 or 1:9 mixture of total bone marrow cells from MxCre;FF1 or SclCre;FF1 mice that have been induced with polyinosine-polycytosine (pIpC) or tamoxifen, respectively, and from wild type mice that express GFP in all hematopoietic lineages (UBC-GFP). This approach allowed us to distinguish mutant (GFP negative) and wild-type (GFP positive) cells by flow cytometry and thus quantify the contribution of the JAK2-V617F cells to different lineages. *Results.* Recipients transplanted with a 1:1 ratio of mutant and wild type bone marrow cells developed a PV phenotype 4 weeks post-transplantation. At the same time we observed a replacement of wild type cells by the JAK2-V617F cells in the peripheral blood. This displacement was most rapid in erythroid lineage (>95%). After 24 weeks the hemoglobin returned to normal and the platelet counts showed a strong increase ($>5\times10^9$/L), although the percentage of JAK2-V617F positive cells remained high in all lineages. Recipients transplanted with a 1:9 ratio of mutant and wild type bone marrow also showed a PV phenotype that later switched to ET, but the timing was delayed by 8-10 weeks. Sections of the long bones and sternum revealed the presence of fibrosis in all mice transplanted with JAK2-V617F bone marrow cells in 1:1 and 1:9 ratios. When the bone marrow cells harvested during the ET phase were transplanted into secondary recipients, the PV phenotype did not reappear and the ET phenotype persisted during the entire follow-up (up to 44 weeks post transplant). The fraction of Lin-, Sca+ c-kit+ (LSK) cells was increased 8-10 fold in MxCre;FF1 mice expressing JAK2-V617F, but LSK cells from these mice induced a MPN phenotype in only a fraction of lethally irradiated recipients. *Summary.* In our transplantation model, JAK2-V617F bone marrow cells rapidly outcompeted the wild type cells when transplanted in 1:1 and 1:9 ratios and reproduced the PV phenotype of the donor mice. The LSK stem cell compartment is expanded in JAK2-V617F expressing mice, but the reconstitutive potential of these LSK cells may be diminished.

0398

CHARACTERIZATION OF A KANK1-PDGFRB FUSION IN AN IMATINIB-RESPONSIVE MYELOPROLIFERATIVE NEOPLASM WITH SEVERE THROMBOCYTHEMIA

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Myeloproliferative neoplasms (MPNs) are clonal stem cell disorders characterized by mature myeloid cell overproduction. Among them, essential thrombocythemia is associated in 50% of cases with the JAK2 V617F mutation. Mutations in the thrombopoietin receptor, which activates JAK2, were also found in this MPN. Here, we analyzed a patient presenting clinical features suggestive of an essential thrombocythemia but in which JAK2 V617F mutation and BCR-ABL fusion were excluded. The karyotype suggested a previously unreported t(5;9)(q32;p24) translocation. By combining fluorescence *in situ* hybridization (FISH) and PCR, we identified a KANK1-PDGFRB fusion transcript. KANK1 (also called ANKRD15) is a proposed tumor suppressor gene in renal cell carcinoma. Upon imatinib mesylate treatment (100 mg/day), the patient achieved hematological remission. The KANK1-PDGFRβ protein comprises three coiled coil domains of KANK1 fused in frame to the fifth Ig-like domain and the kinase domain of PDGFRβ. When expressed in Ba/F3 cells, the fusion protein was phosphorylated on tyrosine residues, induced the activation of STAT5 and enabled cytokine-independent cell growth like other activated PDGFRβ fusion proteins. Deletion of the fifth Ig-like domain did not affect the transforming ability of the protein, suggesting that this unusual translocation breakpoint in PDGFRB is not associated with any proliferative advantage. PDGFRβ translocation partners are characterized by the presence of dimerization domains that enable the constitutive activation of the PDGFR tyrosine kinase activity. To assess whether KANK1 coiled coil domains are implicated in KANK1-PDGFRβ transforming activity, we generated mutants in which one or more of these dimerization domains were deleted. KANK1 coiled coil domains are important but not sufficient to induce a transforming activity when fused to the PDGFRβ. This is the first report of severe thrombocythemia associated with a PDGFRB translocation, without prominent eosinophilia. This is in sharp contrast with the reported clinical features of MPNs associated with PDGFRB rearrangements, which often include eosinophilia and thrombocytopenia. Therefore, we speculate that the fusion partner KANK1 plays a role in the disease, in line with a previous report showing that it is down-regulated in JAK2 V617F-positive MPNs.

0399

UPREGULATION OF HUMAN TRIBBLES HOMOLOGUE 3 (TRB3) BY ANAGRELIDE: A NOVEL MOLECULAR MARKER FOR THE DISCOVERY OF CANDIDATE PLATELET LOWERING AGENTS

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Background and Aims. TRB3, a mammalian orthologue of Drosophila Tribbles is a member of an emerging group of kinase-like proteins, increasingly implicated in the modulation of signal transduction pathways and gene transcription. TRB3 has been reported to inhibit AKT and MAP kinase cascades, both of which are involved in TPO-stimulated signalling. Accordingly, in an attempt to expand our understanding on the molecular mechanisms that regulate megakaryocytopoiesis and to clarify further how this process is affected by the platelet lowering agent anagrelide, we investigated whether this drug modulates the expression of TRB3. *Methods.* Human umbilical cord blood-derived $CD34^+$ cells were expanded in IMDM-based serum free medium supplemented with haematopoietic growth factors and then induced to undergo megakaryocytic differentiation by further culture with TPO. Relative expression levels of selected transcripts were quantified by Real Time PCR using gene-specific probes. *Results.* Culture of haematopoietic cells with TPO resulted in a time and dose-dependent decrease in the mRNA levels of TRB3, with a maximal effect (80% reduction) observed at 40 ng/mL TPO after four days incubation. Addition of anagrelide at the beginning of the differentiation period counteracted this effect of TPO, resulting in a time and dose-dependent increase of TRB3 levels (EC_{50} = 15 nM, maximal increase of ~4-fold after 8 days with 1 μM anagrelide). These changes were mirrored by reciprocal changes in the expression of the megakaryocyte-specific gene GpIIb. Anagrelide has anti-phosphodiesterase III (PDEIII) activity. However, the equipotent PDE III inhibitor cilostamide, had no discernible effect on TRB3 expression. Screening of small chemical entities for their ability to increase TRB3 mRNA expression predicted their ability to inhibit megakaryocyte development in a conventional growth assay. *Conclusions.* These findings point out to a novel mechanism for the inhibition of platelet production and suggest that TRB3 could be used as a novel marker to assist in the identification of candidate anti-megakaryocytic agents.

0400

JAK2 V617F PROMOTES EXPRESSION OF ONCOSTATIN M (OSM) IN MYELOID PROGENITOR CELLS: A POTENTIAL LINK BETWEEN ABNORMAL JAK-SIGNALING AND BONE MARROW ANGIOGENESIS AND -FIBROSIS IN MYELOPROLIFERATIVE NEOPLASMS

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Background. Polycythemia vera, idiopathic myelofibrosis, and essential thrombocythemia are a group of myeloproliferative neoplasms (MPN) characterized by gain-of-function mutations in the Janus kinase 2 (JAK2) gene. A majority of the MPN patients carries a point mutation at codon 617 (V617F) which is located within the autoinhibitory pseudokinase domain and leads to constitutive activation of the kinase. A causal role for JAK2 in MPN has been proposed based on mouse transplantation models in which Jak2 V617F produces a condition reminiscent of MPN. Moreover, a number of *in vitro* studies have implicated the activated JAK2 mutant in cell growth and survival. However, the mechanisms driving growth and differentiation of hematopoietic cells by the JAK2 mutant remain largely unknown. It is also unclear whether JAK2 V617F contributes to the marked bone marrow microenvironment alterations, notably the increased angiogenesis and fibrosis observed in patients with MPN. Oncostatin M (OSM), a cytokine of the

IL-6 family has been implicated as a growth factor triggering the proliferation of various mesenchymal cells including endothelial cells and fibroblasts. Moreover, OSM has been described to be involved in megakaryocytic maturation and in erythropoietic differentiation. *Aims.* The aim of the current study was to investigate the expression and functional role of OSM in MPN. *Methods.* Expression of OSM in the bone marrow of MPN patients was investigated by immunohistochemistry and real time PCR. To investigate the role of JAK2 in expression of OSM, Ba/F3 cells with doxycycline-inducible expression of wild type JAK2 or JAK2 V617F were generated. In addition, the human JAK2 V617F+ cell lines HEL, SET2, and UKE1 were used. *Results.* As assessed by immunohistochemistry performed on bone marrow sections of patients with MPN (n=15), megakaryocytes and myeloid progenitors expressed the OSM protein at high levels. JAK2 V617F-positive MPN patients were found to express significantly higher OSM mRNA levels than normal bone marrow samples derived from patients undergoing lymphoma staging (Figure 1). Interestingly, expression of OSM was not increased in BCR/ABL-positive CML patients (Figure 1). In Ba/F3 cells, doxycycline-inducible expression of JAK2 V617F led to a substantial upregulation of OSM mRNA and protein levels, whereas expression of wild type JAK2 or BCR/ABL did not promote expression of OSM. Correspondingly, the JAK2 V617F-positive human cell lines HEL, SET2, and UKE1 were found to express significantly higher levels of OSM than the BCR/ABL-positive K562 cell line and knockdown of JAK2 V617F downregulated OSM expression in these cell lines. Finally, STAT5 was found to be involved in JAK2 V617F-dependent expression of OSM since RNAi knockdown of STAT5 substantially reduced OSM expression levels and a constitutively activated STAT5 mutant upregulatd OSM expression. *Conclusion.* Together, our data show that neoplastic cells in MPN express OSM in a JAK2 V617F- and STAT5-dependent manner. Whether JAK2 V617F-dependent OSM expression is involved in growth and differentiation of neoplastic cells and/or in remodelling of the bone marrow microenvironment in patients with MPN is currently under investigation.

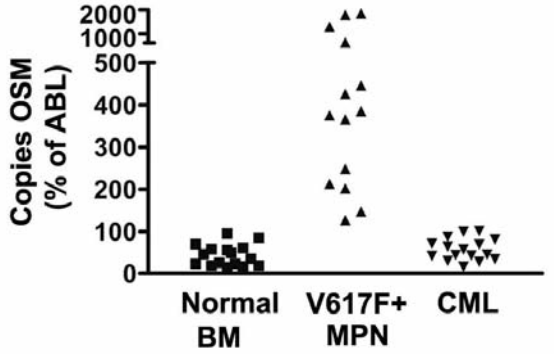

Figure 1. Expression of OSM in the bone marrow.

0401

THE ROLE OF THE JAK2 GGCC HAPLOTYPE, TET2, AND CBL IN FAMILIAL MYELOPROLIFERATIVE NEOPLAMS

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Background. Myeloproliferative neoplasms (MPN) encompass distinct hematologic malignancies that share common pathogenetic features such as the *JAK2*-V617F mutation. Recent reports showed that about 80% of *JAK2*-V617F mutations occur on a particular haplotype of the *JAK2* gene, namely the GGCC or 46/1 haplotype. It has been hypothesized that this haplotype might explain familial clustering in MPN. If this assumption is correct, higher frequency of the GGCC haplotype is expected in familial MPN when compared to sporadic cases. To test this hypothesis, we investigated the role of the GGCC haplotype in a large series of patients with familial and sporadic MPN from the same demographic area in Italy. In order to evaluate additional genetic factors that might underlie familial MPN, we examined the presence of germline *TET2* and *CBL* mutations. *Methods.* A total of 108 familial MPN patients (53 pedigrees) were identified through interview-based investigation of 960 sporadic MPN patients. The HapMap Tuscani in Italia population (TSI) was used as a demographic control. Molecular studies were performed in familial cases with available DNA from granulocytes and T lymphocytes (88 of 108) and in 123 sporadic MPN patients. A tagging single nucleotide polymorphism (SNP; rs10974944) was used for determining the *JAK2* haplotype. *Results.* Association analysis revealed a significant correlation between the occurrence of *JAK2*-V617F and the GGCC haplotype in patients with familial and sporadic MPN when compared to the TSI population. The GGCC haplotype frequency was higher in familial (0.44) and sporadic MPN patients (0.49) than in the TSI population (0.30). The risk of acquiring *JAK2*-V617F was higher for patients heterozygous or homozygous for the GGCC haplotype in familial ($P=0.0077$, OR 2.33, 95% CI 1.24-4.36) as well as sporadic MPN ($P=2.6\times10^{-05}$, OR 3.68, 95% CI 1.95-6.97) when compared to TSI. However, familial MPN did not differ significantly from sporadic MPN in disease risk conferred by the GGCC haplotype. Twelve TET2 mutations were detected in granulocytes from 11 of 88 patients. Of these, 7 were somatic TET2 mutations, whereas 5 patients displayed the same mutation in T lymphocytes: two patients carried a non-annotated SNP (P1723S), one a normal variant (V1718L) and two patients had new germline mutations (R1440Q and A241V). The TET2 R1440Q mutation was absent in the other affected member of the same pedigree, thus excluding segregation of this mutation with the disease. In case of the A241V mutation, no DNA was available from the other affected family member. No germline CBL mutations were found. *Conclusions.* Our results show no significant difference between familial and sporadic MPN in the risk of acquiring *JAK2*-V617F conferred by the GGCC haplotype. Thus, the GGCC haplotype does not explain familial clustering in MPN. Mutations in TET2 and CBL genes were recently suggested to play a role in MPN pathogenesis. However, we could not identify germline mutations that segregate with the disease phenotype. In conclusion, we could disprove the hypothesis that the *JAK2* GGCC haplotype and TET2 and CBL genes play a role in familial MPN. Thus, the genetic basis of MPN predisposition still remains to be defined.

0402

MOLECULAR MECHANISMS OF C-CBL MUTATIONS IN MYELOID NEOPLASMS

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C-CBL proto-oncogene is the cellular homolog of the v-Cbl transforming gene isolated from the Cas NS-1 murine leukemia virus that causes B-cell lymphoma and myeloid leukemia in mice. Recently, we and other groups reported that c-CBL is mutated in myeloid neoplasms, especially in myelodysplastic/myeloproliferative neoplasms with acquired 11q-UPD (uniparental disomy). C-CBL is thought to be involved in the negative modulation of tyrosine kinase signaling, primarily through ubiquitin-mediated down-regulation of activated tyrosine kinases, depending on its E3 ubiquitin ligase activity. According to this functionality, C-CBL could act as a tumor suppressor, which is also supported by the fact that C-CBL null mice developed spontaneous cancers in complete penetrance. On the other hand, when transduced into NIH3T3 fibroblasts, tumor-derived C-CBL mutants thus far tested showed clear oncogenic potentials in terms of anchorage-independent growth in soft agar and tumor formation in nude mice. So to clarify the molecular mechanisms of mutant C-CBL-induced oncogenicity, we further characterized behavior of various mutants of tumor-derived C-CBL proteins. As previously reported, tumor-derived C-CBL mutants not only have defective E3 ubiquitin ligase activity but also inhibits that of wild-type C-CBL, leading to prolonged activation of receptor tyrosine kinases after ligand stimulations. C-CBL mutants that lacked their C-terminal ubiquitin associated/leucine zipper (UBA/LZ) domain were no more able to be dimerized but still showed transforming activity in NIH3T3 cells. While all tumor-derived C-CBL mutants were strongly phosphorylated, these UBA/LZ domain-deleted mutants remained to be unphosphorylated. Thus, neither dimerization nor phosphorylation seemed to be required for transforming capacity. On the other hand, when introduced a mutation that abolishes their binding to phosphorylated tyrosine kinases, these tumor-derived C-CBL mutants were no more transform NIH3T3 cells, suggesting that binding to phosphorylated tyrosine kinases are essential for their transforming capacity. Finally tumor-derived C-CBL mutants were introduced with additional mutations at tyrosine residues, Y700, 731 and 774, which are the major tyrosine phosphorylation sites in wild-type C-CBL, and were examined their effects on transforming capacity. The tumor-derived C-CBL mutants having Y700F/Y731F/Y774F still retained transforming potentials in NIH3T3 cells, although the transformation of NIH3T3 cells with

these mutants seemed to be weaker. Thus, C-terminal domains are not essential for oncogenic capacity of tumor-derived C-CBL mutants. Our results indicated that oncogenic potential of tumor-derived C-CBL mutants seemed to primarily depend on defective E3 ubiquitin ligase activity, while the impact of C-CBL's functions as a signal transducer on the transforming potentials of tumor-derived C-CBL mutants are unremarkable and could be dispensable.

0403

TELOMERE LOSS IN PH - NEGATIVE CHRONIC MYELOPROLIFERATIVE NEOPLASMS: THE ROLE OF JAK2 V617F MUTATION ALLELE BURDEN AND THE INFLUENCE OF THE HISTORY OF THE ILLNESS

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Telomeres are reliable indicators of previous cell proliferation and cell ageing. A loss of telomere length (TL) has been observed in patients with Ph-negative Chronic Myeloproliferative Neoplasms (Ph-neg-CMNs) (Ferraris AM et al, Br J Haematol 2005; Bernard L et al, Leukemia 2009). This supports the possible influence of TL in the development of chronic myeloid disorders. Moreover, TL might be of prognostic relevance in these disorders. *Methods.*Peripheral blood (PB) samples were obtained from 115 Ph-neg-CMNs patients (median age 64 yrs, range 10-89): 60 had PV and 55 ET. JAK2^{V617F} mutation was present in 53 of 55 (96%) evaluable PV patients and in 29 (53%) ET patients. Sixty-five patients had been exposed for at least one year to various treatments, 61 received hydroxyurea. Forty-seven patients had never been exposed to cytotoxic drugs. Samples were obtained either at diagnosis or during follow-up. As a control, PB samples from 104 healthy age-matched subjects (median: 61 yrs, range 49-102) were analyzed as well. TL was assessed by Southern blot analysis. JAK2^{V617F} mutation was detected by ASO-PCR and digestion with BSAXI. *Results.*PV and ET patients showed individual progressive TL shortening correlated with age as observed in the healthy population. However, PV-ET patients had TL significantly shortened (6,000 bp, range: 3090-9770) compared to healthy age-matched individuals (median: 7,086 bp, range: 4926 - 10974) (Figure 1).

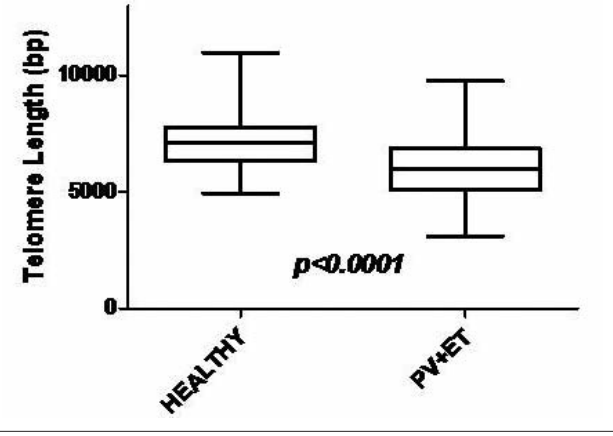

Figure 1. Comparison of TL among age-matched groups of healthy subjects, polycythemia vera patients, and essential trombocytopenia.

The most pronounced TL loss was detected in PV, with TL values significantly shorter (median TL: 5,405 bp, range: 3090 - 9570) compared to ET (median TL: 6,540 bp, range: 4,170-9,770) (P=0.0012). At univariate analysis, short TL correlated significantly with JAK2^{V617F} mutation allele burden >50% (P=0.0003). In multivariate logistic regression analysis, PV diagnosis and JAK2^{V617F} omozigosity remained independently associated with TL reduction. On the other hand, we were unable to detect any correlation between TL reductions and other clinical parameters, including sex and thrombotic complications. Disease duration had a clear impact on telomere loss, with significant differences in TL between patients with short disease duration (6,310 bp, range 3,360-9,770) compared to age-matched patients at >32 mos. since diagnosis (5,460 bp, range 3,090-9,570) (Figure 2). Accordingly, the chronic exposure to cytotoxic drugs, especially to HU, was associated with TL loss (5,941 bp range: 3,880-7,950) compared to age-matched untreated patients (6,560 bp, range: 3,430-9,570) (P=0,03). *Conclusions.* i.ET and PV are confirmed to present with TL reduction compared to the age-matched healthy population; ii.also high JAK2^{V617F} mutation burden was strongly correlated to TL loss; iii.PV has markedly shorter TL compared to ET and this likely reflects the differences in JAK2^{V617F} mutation burden between these two disorders; iv.the duration of the disease along with the need of cytotoxic treatments produce accelerated TL erosion.Although the role of the natural history of Ph-neg-CMNs may play an important role on TL shortening, the results emphasize the need of cautious use of cytotoxic drugs and raise concern about their prolonged use. Further studies are warranted to identify drugs targeting the mutated JAK2^{V617F} gene or other effective agents with reduced toxicity and low induction to cell senescence.

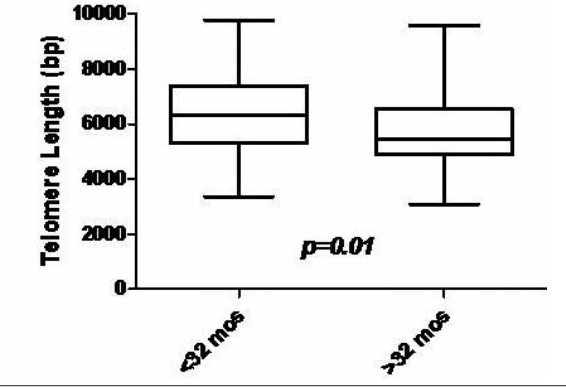

Figure 2. Differences in TL in PV-ET patients according disease duration.

0404

IDENTIFICATION OF ONCOSTATIN M AS A STAT5-DEPENDENT MEDIATOR OF BONE MARROW REMODELING IN KIT D816V-POSITIVE SYSTEMIC MASTOCYTOSIS

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Background. Systemic mastocytosis (SM) is a neoplastic disease of mast cells (MC) and their bone marrow-derived progenitors. The clinical picture in SM is variable ranging from an indolent course to highly aggressive variants with short survival. The pathologic hallmark in SM is the multifocal dense infiltrate of neoplastic MC in the bone marrow. Moreover, marked alterations of the marrow microenvironment are noticed in most patients. These include increased angiogenesis, osteosclerosis and sometimes massive fibrosis. In a majority of patients, MC display the KIT mutation D816V which leads to ligand-independent signaling of the KIT receptor. However, so far, little is known about KIT D816V-dependent expression of molecules in neoplastic MC and their role in the pathogenesis of mastocytosis. Oncostatin M (OSM) is a pleiotropic cytokine belonging to the interleukin-6 family that can elicit different biological effects depending on the disease and target cell type. OSM has been shown to act as a growth factor for mesenchymal cells including endothelial cells, fibroblasts, and osteoblasts and has recently been implicated in the generation and maintenance of a proper microenvironment in hematopoietic tissues. *Aims.* In the current study we examined expression and potential role of OSM in neoplastic mast cells. *Methods.* Expression of OSM in the bone marrow of patients with SM was determined by immunohistochemistry. To investigate the role of KIT in expression of OSM, Ba/F3 cells with doxycycline-inducible expression of wild type KIT or KIT D816V as well as the HMC-1 mast cell line were used. *Results.* As assessed by immunohistochemistry performed on bone marrow sections of patients with SM (n=18), typical spindle-shaped neoplastic MC were found to express OSM. Serial section-staining confirmed that tryptase-positive MC co-express OSM. In Ba/F3 cells, doxycycline-inducible expression of KIT D816V led to a substantial upregulation of OSM mRNA and OSM protein, whereas expression of wild type KIT did not affect expression of OSM. In addition, the KIT D816V-positive mast cell line HMC-1 was found to express OSM at high levels, whereas a KIT D816V-negative

HMC-1 subclone expressed only baseline levels of OSM. Correspondingly, the KIT D816V-targeting drug midostaurin as well as RNAi knockdown of KIT expression decreased expression of OSM. Knockdown of STAT5 expression in HMC-1 and KIT D816V-expressing Ba/F3 cells was found to substantially reduce expression of OSM whereas expression of a constitutively activated STAT5 mutant upregulated the expression of OSM. To investigate the effect of MC-derived OSM on cells of the bone marrow microenvironment, human microvascular endothelial cells, human immortalized osteoblasts, and primary human bone marrow fibroblasts were incubated with cell culture supernatants from HMC-1 cells. As assessed by 3H-thymidine incorporation assay, supernatants from KIT D816V-positive HMC-1 cells were found to stimulate growth of these cells. This growth stimulatory effect was inhibited by preincubation of supernatants with a neutralizing anti-OSM antibody. *Conclusion*. Together, our data show that neoplastic mast cells express OSM in a KIT D816V- and STAT5-dependent manner. MC-derived OSM stimulates growth of mesenchymal cells potentially involved in angiogenesis, fibrosis, and osteosclerosis in the bone marrow. Thus, OSM could represent a novel key molecule and potential target in the pathogenesis of systemic mastocytosis.

0405

DOWNSTREAM EFFECTORS OF FIP1L1-PDGFRA AS TARGETS FOR THERAPY IN CHRONIC EOSINOPHILIC LEUKEMIA

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Background. Chronic eosinophilic leukemia (CEL) is a rare hematological malignancy characterized by overproduction of eosinophils and is frequently caused by the FIP1L1-PDGFRA fusion gene. The small molecule tyrosine kinase inhibitor (TKI) imatinib is very active against FIP1L1-PDGFRα positive CEL, but the development of resistance, therapy related side effects and required lifelong treatment encourage the development of additional therapeutic approaches. *Objectives.* This study aimed at the identification of specific downstream effectors of FIP1L1-PDGFRα that could potentially be used as additional therapeutic targets and/or diagnostic markers in CEL. *Methods.* Gene expression patterns of untreated and TKI treated human FIP1L1-PDGFRα positive EOL-1 cells were compared. The importance of the identified genes for the proliferation and survival of EOL-1 cells was further explored using RNAi technology and specific inhibitors. In addition; their expression was examined in CEL patients. *Results.* We found 51 genes to be differentially expressed in EOL-1 cells upon treatment with both imatinib and sorafenib. Fourteen significantly up- or downregulated genes were selected and we could demonstrate a modulation of their expression by BCR-ABL1 and FLT3 kinase activity to a similar extent as in the context of FIP1L1-PDGFRα. One of these genes is CCL2 which was 100-fold upregulated downstream of FIP1L1-PDGFRα. CCL2 is a member of the CCβ subfamily of chemotactic cytokines and has a known role in cancer cell proliferation. siRNA mediated knock down of CCL2 expression as well as treatment with an anti-CCL2 neutralizing antibody significantly decreased EOL-1 cell proliferation. This antiproliferative effect on EOL-1 cells was further increased when CCL2 knock down was combined with a low nanomolar dose of imatinib. Addition of CCL2 to the culture medium abolished the siRNA mediated inhibitory effect, indicating that CCL2 production induced by FIP1L1-PDGFRα acts through an autocrine loop. Because CCL2 selectively binds to a G-coupled protein receptor CCR2, we investigated the role of CCR2 in EOL-1 cell proliferation. Blocking of the CCL2 receptor using siRNA technology or a CCR2 antagonist also inhibited EOL-1 cell growth, further demonstrating the role of CCL2 in EOL-1 cell proliferation. In PDGF stimulated NIH3T3 cells, we could confirm that CCL2 is a downstream target of PDGFRα. FIP1L1-PDGFRα positive CEL patients showed a slightly elevated CCL2 expression level compared to FIP1L1-PDGFRα negative hypereosinophilia patients. Follow up of one CEL patient documented highly increased CCL2 and CCR2 levels at time of progression towards an acute eosinophilic leukemia. *Conclusions.* Our results indicate that CCL2 production in EOL-1 cells is upregulated by FIP1L1-PDGFRα kinase activity and that CCL2 stimulates the proliferation of EOL-1 in an autocrine way. As such, CCL2 may represent a novel target for therapy in FIP1L1-PDGFRα positive CEL. Currently several CCL2 targeting compounds are already being tested for solid tumors. Further research is currently ongoing to elucidate the pathway employed by CCL2 and its receptor CCR2 to stimulate EOL-1 cell proliferation and to better characterize its function in FIP1L1-PDGFRα positive disease.

0406

FIP1L1-PDGFRA CAN CAUSE BOTH MYELOPROLIFERATIVE AND LYMPHOPROLIFERATIVE DISEASE IN MOUSE BONE MARROW TRANSPLANT MODELS

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Background. FIP1L1-PDGFRA is a fusion tyrosine kinase that causes chronic eosinophilic leukemia (CEL), a myeloproliferative disease that mainly affects the esoinophil lineage in humans. In mice, FIP1L1-PDGFRA has been described to lead to a myeloproliferative disease with a dramatic expansion of granulocytes ($Gr1^+/Mac1^+$). Eosinophilia was also observed in mouse models of FIP1L1-PDGFRA induced disease when FIP1L1-PDGFRA expression was combined with IL5 expression. Although FIP1L1-PDGFRA predominantly is found in patients with CEL, it has also been detected in a subset of patients with lymphoblastic T-cell lymphoma. *Aims.* We have set up mouse bone marrow transplant models to determine if FIP1L1-PDGFRA can also induce a lymphoproliferative malignancies in mice. *Methods.* Lineage depleted (Lin^-) bone marrow cells were transduced with retroviral vectors containing the FIP1L1-PDGFRA gene and a GFP reporter gene. These cells were transplanted into sublethally irradiated mice (10^6 cells/mouse). *Results.* Three weeks after transplantation, most mice developed leukocytosis mostly consisting of GFP^+ granulocytes. Despite the elevated blood counts, most animals survived and five weeks after transplantation most of the GFP^+ granulocytes had disappeared. Eleven weeks after transplantation mice developed enlarged lymph nodes, with a slightly increased GFP^+ cell population in their blood (<20%). In contrast with the earlier detected GFP^+ granulocytes, this GFP^+ population was of the lymphoid lineage. The enlarged lymph nodes contained >80% GFP^+ $CD4^+/CD8^+$ or a combination of $CD4^+/CD8^+$ and $CD4^-/CD8^+$ T-cells. In addition to the lymphomas, some animals developed splenomegaly and a lymphoproliferative phenotype with an increase of GFP^+ cells in blood and bone marrow. The GFP^+ (FIP1L1-PDGFRA+) T-cells were sensitive to sorafenib, a kinase inhibitor shown to inhibit FIP1L1-PDGFRA kinase activity, illustrating that these cells were dependent on FIP1L1-PDGFRA kinase activity for their proliferation and survival. *Conclusions.* We have shown that mice transplanted with FIP1L1-PDGFRA transduced Lin- cells can develop both myeloproliferative disease and T-cell lymphomas.

0407

JAK2V617F ALLELE BURDEN IS ASSOCIATED WITH APOPTOSIS-RELATED GENES EXPRESSION IN ESSENTIAL THROMBOCYTHEMIA

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Background. Essential Thrombocythemia (ET) is a Chronic Myeloproliferative Disorder (MPD) characterized by an increase in the platelet count associated with bone marrow megakaryocyte hyperplasia. The JAK2V617F mutation is present only in 50-60% of ET patients. Therefore, the physiopathology of this disease remains unknown. It seems that apoptotic machinery deregulation contribute to ET pathogenesis. *Aims.* To determine the apoptosis-related genes expression: the anti-apoptotic a1, mcl-1, bcl-2, bcl-xL and bcl-w and the pro-apoptotic bax, bid, bik, bok, and bimEL in $CD34^+$ hematopoietic stem cells (HSC) and leukocytes from ET patient and to correlated these data with JAK2V617F allele burden. Subjects and *Methods.* We evaluated 53 controls (23 males and 30 females; mean age of 45.2 years) and 26 ET patients (5 males and 21 females; ma=60.2y). The JAK2V617F allele burden was determined by real time allelic discrimination PCR assay. Bone marrow $CD34^+$ HSC were separated using Ficoll-Hypaque protocol followed by Miltenyi CD34 isolation kit and peripheral leukocytes was obtained by Haes-Steril method. Total RNA from CD34+ HSC and leukocytes was extracted according to TrizolTM method, High Capac-

ityTM Kit was used to synthesize cDNA and apoptotic-related genes were quantified by real time PCR. The gene expression results were given as 2-ΔΔCt and statistical analyses were performed by Mann-Whitney and Spearman tests. *Results.* In CD34+ HSC, anti-apoptotic a1 and pro-apoptotic bid, bik, and bok mRNA levels were increased in ET patients (median=32.11, 4.10, 2.20 and 58.33, respectively) in comparison to controls (0.71, 1.61, 1.99, 0.73, respectively) (P<0.0001, P=0.0191, P=0.0298, P=0.0005, respectively) while pro-apoptotic bax level was found lower (0.22) than in controls (5.68) (P=0.0231). Only a1 gene expression was found elevated in ET patients´ leukocytes (14.73) compared to control group (1.93) (P=0.0015). We also found in leukocytes from ET JAK2V617F positive patients a higher bcl-2 expression (4.23) and a lower bik expression (0.71) in comparison to JAK2 negative patients (0.66, 3.23, respectively) (P=0.0388 and P=0.0274, respectively). In addition, bik expression negatively correlated with JAK2V617F allele burden (r=-0.4017; P=0.0210) in ET. *Conclusions.* CD34+ HSC and Leukocytes from ET patients present a deregulation in Bcl-2 family members´ expression. JAK2V617F mutation status and allele burden are linked to apoptosis-related gene expression. We could speculate that the alterations may contribute to ET pathogenesis. Supported by FAPESP (06/50094-8 and 08/54387-5).

0408

A HIGHLY SENSITIVE, RAPID AND NON-PCR BASED METHOD FOR THE DETECTION OF THE JAK2V617F MUTATION IN CHRONIC MYELOPROLIFERATIVE NEOPLASMS

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Background. The JAK2V617F mutation occurs at high frequency in several Ph-negative chronic myeloproliferative neoplasms (Ph-neg-CMNs), such as Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Myelofibrosis (MF). The molecular analysis of this mutation is mandatory in the diagnostic work up of these diseases. *Aims.* Aim of the study is to develop a non-PCR method, based on Allele Specific-Loop mediated isothermal AMPlification (AS-LAMP), for the identification of the JAK2V617F mutation. *Methods.* We have developed a LAMP reaction which efficiently produces a large amount of amplified DNA which is both visible to the naked-eye and monitorable in Real-Time turbidimetry thanks to pyrophosphate salts produced as a result of the DNA amplification process. To ensure selectivity a Peptide Nucleic Acid (PNA) probe specific for the wild-type allele was added thus resulting in absence of normal allele amplification within the reaction time (60 minutes). The AS-LAMP assay was optimized on plasmid controls and by serial dilutions (down to 0.01%) of genomic DNA from a JAK2V617F mutated (HEL) into a JAK2 wild type (K562) cell line. *Results.* We have validated this AS-LAMP assay on DNA obtained from 105 patient samples previously analyzed by conventional Allele Specific PCR (ASO-PCR): 37 PV, 58 TE, 3 IMF, 1 post ET Acute Myeloid Leukemia (AML), 1 post PV and 1 post ET Myelofibrosis, 2 Idiopathic Erythrocytosis (IE) and 2 unclassified CMNs. All samples found positive by ASO-PCR proved also positive when tested by our AS-LAMP assay (100% concordance). When mutant DNA is present in the range of 0.1-100% in wild type DNA, we observed a linear relationship between the mutant allele burden and the amplification time. Accordingly, this assay allows the definition of an allele burden <50% or >50% ("hetero' or 'homozygosity"respectively) and selectively detects the JAK2V617F mutated DNA down to 0.05%. A comparative evaluation with commercial or published quantitative tests is ongoing and will be presented at the meeting. In addition, 6 ET and 1 IE previously found negative by ASO-PCR were found to be low-positive (≤1%) with AS-LAMP and this positivity was confirmed by sequencing analysis after PCR in presence of PNA. None of the negative controls, (1 AML, 2 Acute Lymphoblastic Leukemia, 2 Follicular Non Hodgkin's Lymphoma and 2 Chronic Lymphocytic Leukemia) gave false positive *Results.* We also tested a total of 73 DNA samples from granulocytes obtained from healthy donors: none of them proved positive for JAK2V617F. *Conclusions.* This novel non-PCR based allele-specific LAMP assay is rapid, easy to perform with limited and simple laboratory equipment. It is highly specific, sensitive and significantly increases our ability to detect a low JAK2V617F tumor allele burden. Finally, the assay allows estimation of hetero or homozigosity of the sample. For all these reasons, the AS-LAMP assay can be a valid and powerful tool in the routine molecular monitoring of these diseases.

0409

INCREASED DKK3 PROTEIN EXPRESSION ON PLATELETS AND MEGAKARYOCYTES IN PATIENTS WITH MYELOPROLIFERATIVE NEOPLASMS

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Background. Dickkopf-3 (Dkk3), a secreted member of the dickkopf protein family, has been proposed as a tumor suppressor gene and a new specific marker for tumor endothelial cells. *Aims.* Here we analyzed the expression and function of Dkk3 in platelets and megakaryocytes from healthy controls and patients with BCR-ABL1-negative myeloproliferative neoplasms (MPN). *Methods.* Dkk3 protein and gene expression in platelets was compared to endothelial and blood cell populations by ELISA, real time PCR and immunofluorescence. Moreover, megakaryocytes were isolated from bone marrow aspirates by CD61 microbeads. *Results.* Immunohistochemical studies of Dkk3 expression were performed in 30 MPN, including 10 essential thrombocythemia- (ET), 10 polycythemia vera- (PV), 10 primary myelofibrosis- (PMF), and 10 control reactive bone marrow cases. Highest amounts of Dkk3 protein were found in platelets (150±19 pg/µg total protein) compared to HUVEC (110+8 pg/µg), PBMNC (42+8.4 pg/µg), and CD14+ (15+10 pg/µg), CD8+ (4.2+2.6 pg/µg), CD4+ (3.4+2.7 pg/µg), CD19+ (6.0+8.0 pg/µg) microbead-selected cells whereas Dkk3 in CD15+ and CD235a+ (erythrocytes) cells was not detectable (Figure 1). Moreover, isolated megakaryocytes showed a strong Dkk3 gene and protein expression. By immunofluorescence, Dkk3 co-localized with VEGF in alpha granules of platelets and was released like VEGF upon stimulation with arachidonic acid, TRAP-6 (thrombin receptor activator for peptide 6) and ADP (Adenosine diphosphate). The addition of 100-10000 ng/mL recombinant Dkk3 to whole blood and platelet-rich plasma had no influence on coagulation (APTT, PT, TT) and platelet aggregation. In patients with MPN, we found significantly more Dkk3 positive megakaryocytes than in controls (ET>PV>PMF; 40+10 Dkk3-positive megakaryocytes/mm^2; 31+4; 22 + 3, respectively) compared to control cases (15+3 Dkk3-positive megakaryocytes/mm^2). In control bone marrow sections a weak Dkk3 staining of erythropoietic precursors could be shown. *Conclusions.* Based on our results we conclude that Dkk3 might play a role in the pathogenesis of MPN and plateled-derived Dkk3 released upon stimulation/aggregation like VEGF might affect blood vessel function or maturation.

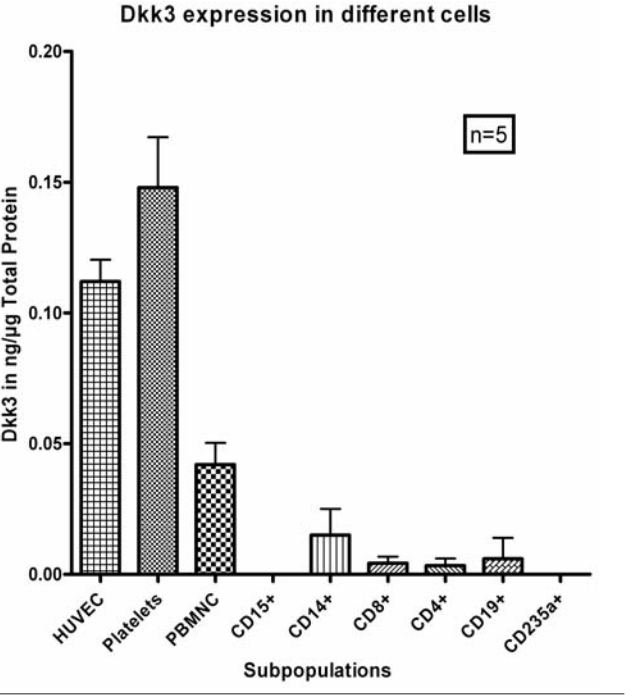

Figure 1. Dkk3 expression in different cells.

Non-Hodgkin lymphomas

0410

COMBINED MODALITY TREATMENT WITH INTENSIFIED CHEMOTHERAPY AND DOSE-REDUCED INVOLVED FIELD RADIOTHERAPY IN PATIENTS WITH EARLY UNFAVOURABLE HODGKIN LYMPHOMA (HL): FINAL ANALYSIS OF THE GERMAN HODGKIN STUDY GROUP (GHSG) HD11 TRIAL

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Purpose. Combined modality treatment consisting of 4 cycles of chemotherapy (CT) followed by involved field radiotherapy (IF-RT) is the standard treatment for early unfavourable HL. In our prior trial for this group of patients (HD8), overall survival (OS) and freedom from treatment failure (FFTF) at 5 years were 91% and 83%, respectively. The HD11 trial thus addressed two major questions: (1) improving outcome by intensifying CT (4xABVD vs. 4xBEACOPPbaseline; Bbas) and (2) defining the best radiation dose (30Gy vs. 20Gy IF-RT). *Patients and Methods.* Between May 1998 and January 2003, 1395 eligible patients aged 16-75 years with untreated early unfavourable stage HL (CS I, IIA with at least one of the risk factors large mediastinal mass (a), extranodal disease (b), elevated ESR (c) or ≥ 3 nodal areas (d); IIB with risk factors c and/or d) were randomized into one of the following 4 treatment arms: 4xABVD + 30Gy (A), 4xABVD + 20Gy (B), 4x Bbas + 30Gy (C) or 4x Bbas + 20Gy (D). Since there are strong indications for an interaction between CT- and RT-doses, a comparison of pooled treatment arms (A+B vs. C+D for comparison of 4xABVD vs. 4x Bbas and A+C vs. B+D for comparison of 30Gy IF-RT vs. 20Gy IF-RT) would be misleading. Therefore all treatment arms were analysed separately. *Results.* Patient characteristics were well balanced between the 4 arms (median age 33 years, 49% male, 6% stage I, 29% B-symptoms). CT- and RT-related acute toxicity occurred significantly more often in the arms with the more intensive therapy (CT: 74.1% vs. 51.8%; RT: 12.3% vs. 5.5%). The complete remission rate 3 months after end of therapy was 94.1% for the whole group and did not differ significantly between the 4 arms. The 5-year estimate of FFTF (primary endpoint) is 85.0% (OS 94.5%, PFS 86.0%). Bbas is more effective than ABVD if followed by 20Gy IF-RT (5y-FFTF difference 5.7%, 95%-CI [0.1%; 11.3%]). This effect does not exist in combination with 30Gy IF-RT (5y-FFTF difference 1.6% [-3.6%; 6.9%]). Similar results are observed for the RT-question: After 4 cycles of Bbas, 20Gy is not inferior to 30Gy (5y-FFTF difference -0.1%, 95%-CI [-5.1%; 4.9%]), whereas after 4xABVD, a relevant inferiority of 20Gy cannot be excluded (-4.0% [-9.5%; 1.4%]). *Conclusions.* A reduction of RT dose from 30Gy to 20Gy IF-RT seems to be justified only in combination with Bbas, but not with a less effective chemotherapy such as 4xABVD. Patients will benefit from an intensified CT such as Bbas only in combination with 20Gy IF-RT but not with 30Gy IF-RT.

0411

EZH2 MUTATION IN FOLLICULAR LYMPHOMA

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Background. Recent evidence suggests that epigenetic mechanisms are of particular importance in germinal centre B lymphomas such as Follicular Lymphoma (FL). Aberrant DNA hypermethylation occurs at 8% of promoters and is more frequent than in non-GC related lymphomas. These hypermethylated loci are enriched for stem cell targets of the EZH2, responsible for transcriptional repression via trimethylation of lysine 27 on histone H3 (H3K27me3). EZH2 has been previously linked with direct control of DNA methylation. Furthermore EZH2 has a putative role, not just in gene repression through control of global DNA and Histone methylation, but also in immunoglobulin gene rearrangement in B cell development. Mutations in the catalytic SET domain of *EZH2* were recently shown by Morin and colleagues to occur in 7% of FL suggesting that attenuated H3K27me3 repression may be an important feature of these tumours. *Aims.* In order to assess the effects of EZH2 on FL transformation (t-FL), we compared *EZH2* mutation with EZH2 and H3K27me3 expression and global methylation status in a series of 20 paired FL and t-FL cases. *Results.* DNA from these 40 samples was tested for the presence of mutation at Y641 by genomic PCR-direct sequencing. Heterozygous mutations were detected in 7 of the 20 patients (35%). Four different point mutations were identified leading to conversion of Y to either F, H, N or S. Germline controls for 4 patients all demonstrated wild-type sequence. A mutation was present in both the FL and t-FL samples of 5 patients, was restricted to the t-FL biopsy in one patient and occurred in only the FL sample of the final affected case. *EZH2* mutation at Y641 was confirmed in the DB, Karpas-422, Su-DHL-6 and WSU-DLCL2 cell lines and was identified for the first time in a further 3 lines RL, Su-DHL-4 and Su-DHL-10. Since EZH2 and DNA methyltransferases have been shown to physically interact we also compared methylation data generated using the Illumina Goldengate platform in 10 FL-tFL pairs and showed no discernible difference in DNA-methylation status between mutated and wildtype cases. EZH2 expression was also assessed by immunohistochemical tissue microarray analysis and showed no difference between wildtype and mutated cases. This was in contrast to H3K27Me3 staining which showed a heterogeneous staining pattern with some mutated cases showing a loss or reduction of expression. Summary: *EZH2* mutation is a frequent mutational event in FL-tFL (35%) and can lead to loss or reduced H3K27me3 expression. The lack of a discernible difference in DNA-methylation patterns between patients with and without mutation and the wide variation in immunohistochemical expression of H3K27me3 in FL/t-FL suggests that EZH2 mutation may exert a greater effect through altering H3K27me3 repression than through widespread alterations in global DNA methylation.

0412

IMPROVEMENT IN LIVER FUNCTION FOLLOWING ECULIZUMAB TREATMENT AVERTS NEED FOR LIVER TRANSPLANT IN A PATIENT WITH PNH AND PROGRESSIVE BUDD-CHIARI SYNDROME

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Background. PNH is a life-threatening progressive disease that results in chronic complement-mediated hemolysis of RBCs and hyperactivation and aggregation of platelet cells. Thromboembolism (TE) accounts for 40 - 60% of deaths among PNH patients and leads to life-threatening complications including impaired hepatic function. Uncommon venous thromboses such as Budd-Chiari syndrome (BCS) and cerebral vein thrombosis (CVT) are frequent findings in PNH patients. Eculizumab inhibits chronic hemolysis and has been demonstrated to improve morbidities and to reduce the risk of thrombosis in PNH patients. *Aims.* We evaluated the response to eculizumab treatment in a 25-year-old male with PNH experiencing multiple episodes of TE, progressive BCS, and declining liver function leading to the consideration of transplantation. *History*. The patient was admitted in June 2005 with abdominal pain and distension. CT scan showed splenomegaly and liver with mottled appearance. Paracentesis recovered 2500 mL of citrinous liquid. MRI revealed BCS and thrombosis of the superior sagittal and left transverse cerebral venous sinus. Peripheral blood flow cytometry confirmed the PNH diagnosis. Despite adequate anticoagulation, the patient experienced two episodes of symptomatic suprahepatic thrombosis. BCS progressed, requiring placement of a porto-systemic shunt (TIPS) and splenic embolization to treat severe refractory thrombocytopenia. The patient experienced two TIPS thromboses, progressive liver damage, and 2 episodes of symptomatic pulmonary embolisms, despite receiving adequate anticoagulation. Given progressive deterioration of hepatic function, liver transplantation was considered. Eculizumab was initiated in January 2009 at the approved dosing regime: 600 mg every 7 days for 4 weeks, and 900 mg every 14 days from 5th week onwards.

Results. During eculizumab treatment, intravascular hemolysis (measured by LDH) decreased from an average of 2.9-fold above normal 1-2 months before eculizumab treatment to normal levels and has been maintained for one year. Platelets increased within one week of treatment and this increase has been sustained over 12 months, consistent with improvement in thromboses and hence reduction in platelet consumption. More importantly, hepatic function noticeably improved: elevated transaminases ALT and AST levels were reduced within 1 week of treatment and albumin and CHE concentrations improved to values well within the normal range, averting need for liver transplant. Portal hypertension was reduced, verified by progressive increase in the interval between paracentesis, with no additional paracenteses after 7.5 months of eculizumab treatment. No new thrombotic episodes took place during one year of treatment. Fatigue resolved completely, in spite of minimum change in anemia, allowing the patient to resume a near normal work and social life. Eculizumab has been well tolerated without adverse events. *Conclusions.* This is the first demonstration of improvement in LFT in a patient with PNH with BCS. Sustained blockade of chronic complement-mediated hemolysis with eculizumab reverted progressive systemic and hepatic venous thrombosis allowed recovery from hepatic dysfunction and thus averted the need for liver transplant, and drastically improved quality of life. These results provide solid evidence that eculizumab is the treatment of choice in patients with PNH and BCS unresponsive to anticoagulation.

Table.

Lab Value (normal range)	Days Pre-Eculizumab		Day of First Dose	Days Post-Eculizumab							
	68	33	0	7	56	112	197	239	295	323	365
LDH (U/L) (≤ 480)	1836	921	--	519	565	248	295	339	344	356	298
Platelets (X 10^9/L) (150-400 x 10^9/L)	34	27	23	53	80	53	94	82	62	74	58
Hemoglobin (g/dL) (13.2-16.2 g/dL)	9.2	9.8	10.7	9.7	9.6	9.9	11.3	10.8	10.3	10.3	10.2
AST (U/L) (≤ 40)	78	70	36	18	9	8	20	20	25	25	19
ALT (U/L) (≤ 37)	63	49	24	20	28	16	25	25	28	23	22
Bilirubin Total (mg/dL) (0.2-1.3 mg/dL)	3.6	2.4	3.3	2.3	2.2	1.7	2.1	2.8	1.6	2.0	1.6
Bilirubin D (mg/dL) (<0.3 mg/dL)	1.7	0.9	1.4	1.0	1.0	0.8	0.9	1.2	0.6	0.7	0.7
CHE (U/L) (> 4,400 U/L)	3,572	2,889	--	--	5,135	5,975	6,968	6,533	7,603	7,860	--
Albumin (g/dL)	--	3.47	--	3.54	3.6	--	4.52	4.67	4.68	4.44	4.27

0413

PIM SERINE/THREONINE KINASES AS POTENTIAL THERAPEUTIC TARGETS IN DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL)

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Background. PIM1-3 represent a family of constitutive active serine/threonine kinases regulating cellular proliferation and survival that have been reported to be over-expressed in several human cancers. Their proto-oncogenic role has been demonstrated in several experimental lymphoma models but never systematically analyzed in primary lymphoma biopsies. *Methods.* We have studied expression of PIM1-3 by immunohistochemistry in a large cohort of well-characterized diffuse-large B-cell lymphoma (DLBCL) cases (n=101, stages I-IV, GC-DLBCL=42 as defined by the Hans algorithm), using tissue microarrays. Functional studies were performed in a panel cell lines derived from GC-type and ABC-type human DLBCLs applying previously characterized small molecule PIM inhibitors. *Results.* Whereas in most (91%) cases cytoplasmic expression of PIM1 was observed, only 12 cases showed also nuclear PIM1 staining in >50% of the tumor cells. In contrast to PIM1, we found only modest levels of PIM2 and PIM3 (>10% of the tumor cells) in most of the cases. PIM1 expression significantly correlated with activation of the signal transducers and activators of transcription (STAT3 & STAT5) as assessed by phospho-STAT staining, and with the fraction of actively proliferating cells (Ki-67, MIB1+). Nuclear expression of PIM1 expression correlated significantly with the Ann Arbor stage of the disease. Interestingly, expression of PIM2 and PIM3 correlated with the amount of tumor-infiltrating FOXP+ regulatory T-cells (Tregs). No significant correlations between PIM expression and the status of MYC-, BCL2- and BCL6-genes, EBV-association or between GC- and non-GC derivation were found. Treatment of DLBCL cell lines with two structurally different small molecule inhibitors significantly impaired cellular proliferation. *Conclusions.* Our study strongly suggests that PIM kinases are collaborating oncogenes in a significant fraction of human DLBCL associated with activation of the JAK/STAT pathway. Blocking of cellular proliferation of DLBCL cells by small molecule inhibitors implies that PIM kinases might represent rational therapeutic targets.

0414

INVOLVEMENT OF GRB2 ADAPTOR PROTEIN IN NUCLEOPHOSMIN-ANAPLASTIC LYMPHOMA KINASE (NPM-ALK) MEDIATED SIGNALING AND ANAPLASTIC LARGE CELL LYMPHOMA GROWTH

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Background. Most Anaplastic Large Cell Lymphoma (ALCL) express oncogenic fusion proteins derived from chromosomal translocations or inversions of the Anaplastic Lymphoma Kinase (ALK) gene. Frequently ALCL carry the t(2;5) translocation, that fuses the ALK gene to the Nucleophosmin (NPM1) gene. The transforming activity mediated by NPM-ALK fusion induces different pathways that control proliferation and survival of lymphoma cells. Grb2 is an adaptor protein thought to play an important role in ALK-mediated transformation, but its interaction with NPM-ALK as well as its functions in regulating ALCL signaling pathways and cell growth have never been elucidated. *Aims.* We characterized the interaction of the adaptor protein Grb2 with NPM-ALK. In particular we focused on three aspects: the binding of Grb2 to NPM-ALK in cells, the phosphorylation of Grb2 by NPM-ALK and role of Grb2 in regulating signalling pathways and proliferation of ALCL cells. *Methods.* Human embryonal kidney cells HEK-293T were transfected with different Grb2 and/or NPM-ALK constructs and immunoprecipitation experiments and immunoblot analysis were performed. Grb2 mutated forms and all kinase mutants were generated by PCR-based mutagenesis. Inducible ALK shRNA SU-DHL-1 and TS cells were obtained by co-transduction with pLV-tTRKRAB (TTA) vector and pLVTHM vector containing the H1 promoter ALK-shRNA cassette. Inducible Grb2 shRNA TS cells were obtained by co-transduction with pLVtTRKRAB vector and pLVTHM vector containing the H1 promoter Grb2-shRNA cassette. These cells undergo NPM-ALK or Grb2 silencing when 1 μg/mL of doxycyclin is added to the medium for 72 hours. shRNA Grb2 lentiviruses were obtained by co-transfection of human HEK-293T cell line. To generate Grb2 shRNA-resistant constructs, wild-type or Y160F Grb2 were mutated in 4 bases in the sequence corresponding to the shRNA (Grb2WTINT3/4 and Grb2Y160FINT3/4). Co-culture experiments and proliferation studies were performed. *Results.* In this study we demonstrate that Grb2 binds to active NPM-ALK and is phosphorylated in human ALCL cells. We identified Y160 as major phosphorylation site of Grb2 by NPM-ALK. We found that Y160 of Grb2 is phosphorylated also by other oncogenic fusion tyrosine kinases such as TPR-MET, BCR-ABL and TEL-JAK2, as well as by wild-type receptor tyrosine kinases (RTK), such as ALK and MET. Further, we show that NPM-ALK combined mutations in Y152-156, Y567 and P415-417 almost completely abrogated Grb2 binding and Y160 phosphorylation. Finally, shRNA knock-down experiments showed that Grb2 is essential for the activation of SHP2 in ALCL and is required for sustained ALCL cell growth. *Conclusions.* The down-modulation of Grb2 in ALCL cells strongly impaired cell proliferation, thus suggesting that Grb2 is fundamental for the full activation of a signalling cascade that involves Shc and SHP2 and assures an optimal proliferation of lymphoma cells. Thus, Grb2 could represent a potential target to control cell proliferation in NPM-ALK mediated lymphomas.

0415

A C-MYC INDUCED GENE EXPRESSION SIGNATURE IN HUMAN GERMINAL CENTER B CELLS PREDICTS SUBTYPES OF AGGRESSIVE NON-HODGKIN LYMPHOMA

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Background. Aggressive Non-Hodgkin Lymphoma (aNHL) are a heterogenous group of malignancies derived from germinal centre B (GC

B) cells. Burkitt's lymphoma (BL) is the most homogeneous aNHL entity, characterized by an aberrant expression of the proto-oncogene c-Myc. Recently BL was defined by a specific gene expression signature including c-Myc as one hallmark. Despite an abundant number of cell line investigations and murine models, there is a lack of experimental systems to investigate the role of c-Myc for the transformation of human GC B cells. Therefore we expressed c-Myc in primary tonsillar GC B-cells and monitored expression changes using microarray gene expression profiling. *Aims.* We performed analyses integrating expression profiles from clinical lymphoma samples pointing us to potential mechanisms of disease initiation and progression. Furthermore we asked whether these changes permit to further subgroup aNHL. *Materials and Methods.* Purified human tonsillar CD10+ GC B cells were transfected with a c-Myc expression plasmid (treatment) or empty vector (control). mRNAs from 8 independent treatment-control pairs (8 human tonsils) were was subjected to gene expression profiling (Affymetrix® U133 plus2.0). Gene set enrichment analysis (GSEA) and bioinformatics integration of two large clinical lymphoma micorarray data sets were used to generate a c-Myc gene expression signature. *Results.* Microarray profiled genes were ranked by concordance of their expression levels with those of c Myc in both tonsillar B cells and tumors. Gene set enrichment analysis revealed a strong enrichment of c Myc target genes and a depletion of CD40/NF-κB pathway targets. We defined the c-Myc signature comprising the top c-Myc responding genes as c Myc index. This index stratifies aNHL patients based on the expression of the c-Myc signature genes. The signature is consistently expressed in BL, while its expression varies in DLBCL. In two independent clinical DLBCL microarray data sets the presence of high c-Myc index is significantly associated with a shorter overall survival. *Conclusions.* Our approach integrates two important aspects of cancer research; intervention in experimental model systems and observation on tumor samples. Mimicking aberrant c-Myc expression in GC B cells provided us with insights into downstream molecular pathways affected, confirmed BL as unique disease entity, and yielded a novel prognostic stratification of DLBCL.

0416

EBV-POSITIVE DLBCL OF THE ELDERLY IS A DISTINCT CLINICO-BIOLOGICAL ENTITY WITH POOR OUTCOME IN CHOP-R TREATED PATIENTS

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Background. Diffuse Large B-cell Lymphoma is the commonest aggressive lymphoma. Epstein-Barr virus-positive DLBCL of the elderly (EBV+ DLBCL) is a provisional sub-type predominantly described in Asian populations. *Aims.* Data on EBV$^+$ DLBCL's response to the gold-standard regimen cyclophosphamide, doxorubicin, vincristine, prednisolone with rituximab (CHOP-R) is limited. Detailed studies of biological determinants have not been performed. Our aim was to determine incidence, obtain CHOP-R survival data, and to study viral and biological characteristics of EBV$^+$ DLBCL in an unselected Australian population. *Methods*. We analysed a consecutive cohort of DLBCL patients drawn over a 6 year period. Cases tested were chosen on the availability of tissue but were otherwise unselected. Survival curves were generated, and detailed assays of gene expression, methylation, tissue microarray, DNA sequencing and EBV-specific T-cell immunity were performed. *Results.* In 121 DLBCL cases in immunocompetent Australian patients, we found 9.1% were EBV$^+$, exclusively in those aged over 50 years (11.3% in the over 50 year-old DLBCL population). The majority of patients were Caucasian. In the over 50's receiving CHOP-R, EBV$^+$ DLBCL demonstrated strikingly inferior event-free (P=0.0002) and overall survival versus EBV- DLBCL (P=0.0368), with EBV-positivity an adverse risk factor by multivariate analysis (P=0.017). Previous evidence inferred from EBNA-2 protein expression suggested type II latency was restricted to the immuno-subdominant EBNA1, LMP1 and LMP2 in 70% of cases, with only 30% showing the EBV (type III) latency pattern seen in PTLD. Our data show these inferences are mistaken. Unexpectedly and in contrast to EBV$^+$ DLBCL occurring in the context of immunosuppression (EBV$^+$ DLBCL-PTLD), it displayed a novel type II/III EBV-latency profile, with up-regulation of EBNA3 but down-regulation of EBNA2. As opposed to latency type III, the major EBV-latency promoter C was consistently hyper-methylated. Polymorphisms in regions encoding for known CD8$^+$ T-cell epitopes were observed in the immuno-subdominant EBV proteins EBNA1 and LMP1, but not the immuno-dominant EBNA3. EBV-specific effector T-cells against multiple EBV-latent proteins could be expanded from baseline peripheral blood. *Conclusions.* EBV$^+$DLBCL is clinically and biologically distinct from EBV- DLBCL and EBV$^+$ DLBCL-PTLD. The poor response of EBV$^+$ DLBCL to CHOP-R indicates new approaches targeting the virus are warranted. Our in depth characterization of EBV viral transcription, promoter methylation, viral polymorphism and EBV-specific effector T-cell immunity is likely to have important therapeutic implications. The role of targeted antiviral immunotherapies and/or EBV lytic-induction strategies should be explored.

0417

DETAILED MOLECULAR ANALYSIS OF CLONAL TCRA AND TCRB GENE REARRANGEMENTS IN SÉZARY SYNDROME REVEALS A RESTRICTED TCR REPERTOIRE

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Background. Sézary syndrome is a rare form of cutaneous T-cell lymphoma (CTCL) characterized by the triad of erytroderma, lymphadenopathy and the presence of malignant T-cells in the peripheral blood (PB). The T-cells are clonal in nature as evidenced by clonally rearranged T-cell receptor (TCR) genes. The vast majority of cases are CD3$^+$/CD4$^+$/TCRαβ$^+$. Although the pathogenesis of CTCL remains unknown, there is evidence for involvement of chronic antigenic stimulation in driving T-cell expansion. The complementarity determining region 3 (CDR3) region of the TCR molecule has the highest antigenic specificity and directly binds to the antigenic peptide in the context of HLA. Detailed molecular characterization of Vα- and Vβ CDR3 regions of a large cohort of patients with Sézary syndrome has not been performed and may contribute to the understanding of the etiology of this disorder. *Aims.* To define TCR clonotypes and to establish the preferential usage of specific TCR-Vα and Vβ families in Sézary syndrome. In addition, we aimed to obtain more insight in the pathogenesis by analysis of CDR3 sequences of *TCRA* and *TCRB* genes *Materials and Methods.* In the present study we evaluated the immunophenotypical and molecular data of a series of 28 patients diagnosed with Sézary syndrome with a high tumorload in PB. We performed detailed immunophenotypical analysis using a T-cell antigen panel and a Vβ antibody (Ab) panel. BIOMED-2 PCR-based GeneScan and sequence analysis of *TCRB* gene rearrangements was performed in all PB samples. *TCRA* gene rearrangements were analyzed using a novel multiplex RT-PCR approach. *Results.* A dominant Vβ protein was detected in 7 out of 11 cases. One case demonstrated dual Ab reactivity whereas no Ab reactivity was detected in 3 cases. Clonal *TCRA* and *TCRB* gene rearrangements were detected in all cases. Sequence analysis of clonally rearranged *TCRB* genes showed overrepresentation of Vβ13, Vβ21 and Vβ22 as compared to healthy controls. No shared/restricted CDR3 motifs could be detected. The Vβ-Jβ2/Vβ-Jβ1 ratio in our series of patients was 1.3, which still clearly differs from the overrepresentation of the Jβ2 gene segment in the PB TCR repertoire of healthy adults (Vβ-Jβ2/Vβ-Jβ1 ratio of 2.6). There was no common Vα gene segment usage and our study did not show identical *TCRA* CDR3 amino acid motifs. *Conclusions.* Based on their immunophenotypic and immunogenotypic features, TCR clonotypes demonstrate heterogeneity. The lack of clearly shared CDR3 motifs of *TCRA* and *TCRB* genes indicate that a single common antigen is not involved in the pathogenesis of Sézary syndrome. Nevertheless, the overrepresentation of Jβ gene segment usage and some Vβ gene segments does reveal a restricted TCR repertoire, which might reflect other, yet to be identified TCR clonotypes, that could be present in a larger cohort of patients.

0418

INDUCTION OF CELL DEATH IN ADULT T-CELL LEUKEMIA CELL LINES BY SURVIVIN-RESPONSIVE CONDITIONALLY REPLICATING ADENOVIRUSES.

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Background. Adult T-cell leukemia (ATL) is an aggressive peripheral T-cell neoplasm that develops after long-term infection with the human

T-cell leukemia virus type I (HTLV-1). Although there has been recent progress in chemotherapy for ATL, the prognosis for patients with ATL is still poor. On the other hand, survivin-responsive conditionally replicating adenoviruses (Surv.CRAs) in which expression of the adenoviral early region 1A (E1A) gene is regulated by the survivin promoter, has been demonstrated to treat a variety of cancers in which surviving is upregulated. *Aims.* Because survivin was also overexpressed in ATL, we examined ATL-selective replication and induction of cell death by Surv.CRAs at the possibility of the clinical application in the future. *Methods.* Six cell lines; 2 ATL cell lines and 3 HTLV-1 infected T-cell lines established from patients in our laboratory and MT-2, were tested. As a negative control, activated peripheral blood lymphocytes (PBLs) of healthy subjects were used. These cells were infected with either E1-deleted replication-defective adenoviruses expressing beta-galactosidase (LacZ) for analyzing gene transduction efficiencies and promoter activities or Surv.CRAs expressing enhanced fluorescent green protein (EGFP) for ATL-specific viral replication and therapeutic efficiencies at a various multiplicity of infection. Promoter activity was measured using the galactosidase enzyme reporter assay. The expression of the coxsackie and adenovirus receptor (CAR), important for cell infection by adenoviruses, was tested by flow cytometric analysis. Viral replication was assessed based on percentage of EGFP-positive cells. The cell viability was determined using a colorimetric assay. *Results.* The survivin promoter was strongly activated in 6 cell lines, both ATL cell lines and HTLV-1 infected T-cell lines. Moreover, the expression of CAR was highly upregulated in these cell lines. In contrast, weak activation of the survivin promoter and low expression of CAR were observed in activated PBLs of healthy subjects. Surv.CRAs efficiently replicated and potently induced cell death in 5 out of 6 cell lines compared to minimal viral replication in normal activated PBLs, in which there was no significant cytotoxicity. Although over-expression of survivin in ATL contributes significantly to apoptosis resistance against conventional chemotherapy, the higher activity of survivin promoter in both ATL cell lines and HTLV-1 infected T-cell lines serves as a potential therapeutic target for survivin-dependent apoptosis induction by Surv.CRAs. Unlike the high CAR expression and strong survivin promoter activation, characterizing the other cell lines, only one HTLV-1 infected T-cell line exhibited resistance to Surv.CRA treatment. This unique cell line may of interest in seeking to elucidate the mechanism of resistance to Surv CRAs. *Conclusions*. This is the first report demonstrating that Surv.CRAs form an attractive potential antileukemic agent that could efficaciously and specifically treat ATL.

0419

MIRNAS EXPRESSION PATTERN IN GASTRIC MALT LYMPHOMA

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Background. MicroRNAs are short non-coding RNA sequences that regulate target genes by inducing translational inhibition and cleavage of targeted transcripts. To date, microRNA have been implicated in development, cell differentiation, apoptosis and proliferation. However, their role in gastric MALT lymphoma remains unknown. *Aim.* To analyze the expression pattern of miRNA in patients with gastric MALT lymphoma. 2- to find a distinctive signature according to the presence or absence of the t(11;18)(q21;q21) API/MALT translocation. *Material and Methods.* Total RNA was extracted from gastric biopsies from 9 patients with MALT lymphoma and 2 with chronic gastritis with the Kit MirVanaTM (Applied Biosystems). Median age of patients was 63 years (range, 32-85), the Ann Arbor stage was 44% stage I, 22% stage II and 22% stage IV and the translocation t(11;18)(q21;q21) was present in 5 patients (56%). miRNA expression was profiled in 384 miRNA via Taqman Low Density Array in ABI PRISM 7900. Expression data was normalized with RNU48 and relative quantification was calculated with the 2- Ct method. The data were presented as log10 of relative quantity of target miRNA. Median of chronic gastritis was used as calibrator for all samples. Data were analyzed by means of Significant Analysis of MicroArrays (SAM), Prediction Analysis of MicroArrays (PAM) and Class Comparison methods using BRB array tools version 3.7.0 and TIGR multiexperiment viewer version 4.3. *Results.* We identified genes that were differentially expressed among the two groups using a random-variance t-test, with a median false discovery rate (FDR) of 10%. We found a group of 5 miRNA (miR-142-3p, miR-594, miR-489, miR-222 and miR-429) overexpressed in gastric MALT lymphoma with respect to chronic gastritis (P<0.001). When we compared miRNA differentially expressed between translocation t(11;18) carriers and non carriers, we found also a group of 9 miRNA with a P<0.05 and a FDR of 10%. Comparing the relative expression levels (2-ΔΔCt) according to Ann Arbor stage, we found a significantly higher level expression of miR-203 and miR-555 in both stages I and II with respect to stage IV. *Conclusions.* Our results suggest specific expression profiles of some miRNA in gastric MALT lymphoma. In addition, several of the miRNA differentially expressed allow us to discriminate cases according to API/MALT translocation and also according to Ann Arbor stage.

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0420

SNP RS6457327 ON CHROMOSOME 6P PREDICTS TRANSFORMATION OF FOLLICULAR LYMPHOMA

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Background. A genome-wide association study (GWAS) recently identified a 26kb region on chromosome 6p21.33 as a genetic risk locus for follicular lymphoma (FL) (Skibola et al, Nature Genetics 2009). The variant allele of a single nucleotide polymorphism (SNP), rs6457327 (involving C>A), associated with reduced FL risk which was validated in four independent FL case-control studies from North America and Germany ($P=4.7\times10^{-11}$ across a total of 645 FL cases and 3377 controls). It is not known if this locus is relevant in established disease. *Aims.* This study sought to determine if rs6457327 is associated with FL risk in the United Kingdom (UK) population and whether it impacts on disease outcome. *Methods.* Germline DNA from 221 FL cases was genotyped for rs6457327 by allelic discrimination polymerase chain reaction (AD-PCR) in a retrospective single centre study of patients treated at St Bartholomew's Hospital between 1977 and 2005. Direct sequence analysis showed complete concordance with AD-PCR genotypes on a validation subset constituting 10% of the samples tested. The significance of differences between groups was determined using 2-by-2 tables, crude odds ratios (OR), 95% confidence intervals (CI) and 2-tailed p values (Fisher's exact test). Survival analysis was performed using Kaplan-Meier survival function with log rank test for equality of survival. *Results.* Comparison of rs6457327 genotypes for the study population to those of a UK control population, obtained from the Wellcome Trust Case Control Consortium (n = 2937), demonstrated a significantly reduced risk of FL for cases carrying AA (OR = 0.47, 95% CI = 0.27-0.79, P=0.003) or AA/AC (OR = 0.73, 95% CI = 0.55-0.97, P=0.02) as opposed to the CC genotype. The minor allele frequencies within the study cohort (0.32) and WTCCC control set (0.38) showed similar distribution to case (0.31) and control (0.38) populations from the GWAS study. Although rs6457327 genotype showed no association with overall survival or event free survival in FL, disease transformation occurred more frequently in the presence of the minor allele, AA and AC versus CC (odds ratio = 2.2, 95% CI = 1.2-4.1; P=0.007). Moreover, the minor allele was also associated with shorter time to transformation (P=0.01) (Figure 1).

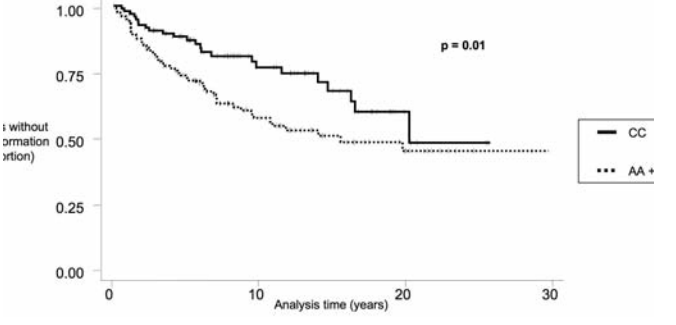

Figure 1. rs6457327 genotype and time to transformation.

Summary/Conclusions. This study confirms a previous report that

found a reduced risk of FL associated with the minor allele for rs6457327 located on chromosome 6p21.33 and demonstrates for the first time that rs6457327 predicts disease transformation. This SNP is located 6 kb telomeric to C6orf15, the only gene located within the 26 kb risk locus. A mouse homologue of this gene has recently been identified as an extra-cellular matrix protein termed emprin. The role of C6orf15 in FL and its transformation is currently under investigation.

0421

CIRCULATING MIR-92A LEVEL IS A NOVEL BIOMARKER FOR MONITORING PATIENTS WITH NON-HODGKIN'S LYMPHOMA

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Backgrounds: The miR 17-92 cluster is a polycistronic miRNA gene and is important in immune cell development and tumorigenesis in lymphoid tissue. Recent studies have demonstrated that circulating miRNAs are detectable in the plasma in various cancer patients. Therefore, the deviations of certain miRNAs detected in plasma of lymphoma patients could be a useful and non-invasive marker to detect cancer development. *Aims.* To evaluate the clinical relevance of the miR-92a in plasma obtained from non-Hodgkin's lymphoma patients. *Methods.* We evaluated plasma miR-92a value (miR-92a/miR-638) using qRT-PCR, in 108 patients with non-Hodgkin's lymphoma, consisting of 66 patients with diffuse large B cell lymphoma (DLBCL), 28 patients with follicular lymphoma (FL), and 14 patients with T-cell lymphoma, at various clinical phases, and compared results with healthy subjects. This study was approved by the institutional review board of Tokyo Medical University (no. 930: approved on June 24, 2008). Total RNA in plasma was extracted as we have previously reported (Tanaka *et al. PLoS One* 4:e5532, 2009). MicroRNAs were quantified by TaqMan® MicroRNA Assays (Applied Biosystems), using microRNA specific stem-loop primers (has-miR-92a, 000431; has-miR-638, 001582; Applied Biosystems). The miR-92a expression was normalized to miR-638 expression. The miR-638 was selected because of its lack of expression variability in plasma. Relative expression was caliculated using the comparative Ct method. *Results.* Plasma miR-92a values in non-Hodgkin's lymphoma were extremely low, compared to healthy subjects (P<0.0001), irrespective of lymphoma sub-type. The expression level of plasma miR-92a in patients with DLBCL was approximately 10% lower than that obtained from healthy volunteers (0.05027±0.01118 versus 0.8355±0.4018: P<0.0001). Of the DLBCL patients, the plasma miR-92a level did not differ according to clinical stage, however, Stage IV patients showed significantly decreased plasma miR-92a levels compared with Stages I to III patients (P<0.0001). Plasma miR-92a in DLBCL patients in complete response (CR) increased significantly compared with the diagnosis level (P<0.0001), but no significant difference was found when compared with healthy individuals (P=0.1167). In the relapse phase, plasma miR-92a levels again decreased and these levels were similar to those at diagnosis (P=0.6936). Similarly, a significantly low level of plasma miR-92a was noted in patients with FL at the time of diagnosis (0.02187±0.01022 versus 0.8355±0.4018: P). It normalized after obtaining CR, and again decreased in the relapse phase. Interestingly, plasma miR-92a level in patients classified as CR uncertain (CRu) tended to decrease compared to that of CR (0.1980±0.07881 in CRu versus 0.5915±0.1881 in CR) (P = 0.0913). The fluctuation pattern of plasma miR-92a detection levels in relation to clinical status in T-cell lymphoma was similar to those of DLBCL or FL. Extremely low levels of the miR-92a value by the qRT-PCR method were found, compared with normal subjects (0.08870±0.06845 versus 0.8355±0.4018: P<0.0001). *Conclusions.* The plasma miR-92a value could be a novel biomarker for monitoring lymphoma patients, especially in those with indolent lymphoma.

0422

CHARACTERIZATION OF COPY-NUMBER ABERRATIONS IN MANTLE CELL LYMPHOMA WITH HIGH-RESOLUTION GENOMIC ARRAYS: CORRELATION WITH GENOMIC COMPLEXITY AND THE PROLIFERATION GENE EXPRESSION SIGNATURE

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Background. Mantle cell lymphoma (MCLs) represents a clinically aggressive lymphoma with a poor prognosis. Genetically it is characterized by the t(11;14)(q13;32) translocation and numerous recurrent secondary copy-number aberrations (CNAs). Gene expression profiling studies have defined a proliferation gene expression signature (PS) where high proliferation scores predict shorter survival. *Aims.* To study CNAs and copy-number neutral loss of heterozygosity (CNN-LOH) in MCL and to correlate specific CNAs with the PS score, genomic complexity and survival. *Methods.* Thirty-one primary MCL cases were investigated using a high-density single nucleotide polymorphism (SNP) array (Affymetrix™ GeneChip® Mapping 250K arrays). Quality control, genotype calling, and probe level normalization were done using the Affymetrix GeneChip® Genotyping Analysis Software (GTYPE) 4.1. Subsequent copy number analysis was performed using the Nexus software from Biodiscovery. Gene expression profiling was performed with Affymetrix™ expression arrays, and the PS score was calculated using the expression levels of 20 proliferation-associated genes as previously described. PS below or above the median (0.99) was considered low or high, respectively. *Results.* The majority of recurrent CNAs identified in this study had previously been described in MCL, and include losses at 1p (55%), 11q (55%), 17p (29%), 9q (29%), 8p (29%), 9p (26%), 6q (23%) and gains at 3q (39%), 8q (26%), 15q (23%), 18q (23%). However, several novel recurrent CNAs were identified, including losses at 10q (13%), 14q (23%), 18p (13%), 20q (16%) and 22q (16%). Notably, the loss at 20q13.2 targeted the SALL4 gene in 4/5 cases. Novel gains were identified at 12p (13%), 16q (13%), 18p (13%) and 22q (13%). Interestingly, some CNAs were associated with increased genomic complexity, for instance, 8p loss correlated with increased average number of CNA regardless of size (P <0.001), whereas losses at 13q and 17p were associated only with increased CNAs >5 Mb (P<0.001 and P=0.03). Furthermore, gains at 7p correlated with high PS score (P=0.02), as did losses at 9q (P=0.037). There were four recurrent chromosomal regions affected by CNN LOH; two at 12q, one at 19p and one at 22q, each identified in two cases. Large 13q losses correlated with improved survival (P<0.05) as did losses at 19p (P<0.05), while gains at 19p tended to correlate with inferior survival (P=0.07). Summary: This high-resolution genomic analysis identified novel recurrent CNAs and CNN-LOH regions and found that several common secondary CNAs correlated with genomic complexity (losses at 8p, 13q, 17p), or proliferation status (7p gains and 9q losses).

0423

BORTEZOMIB MODULATES SURFACE CD20 IN B-CELL MALIGNANCIES AND AFFECTS RITUXIMAB-MEDIATED COMPLEMENT-DEPENDENT CYTOTOXICITY

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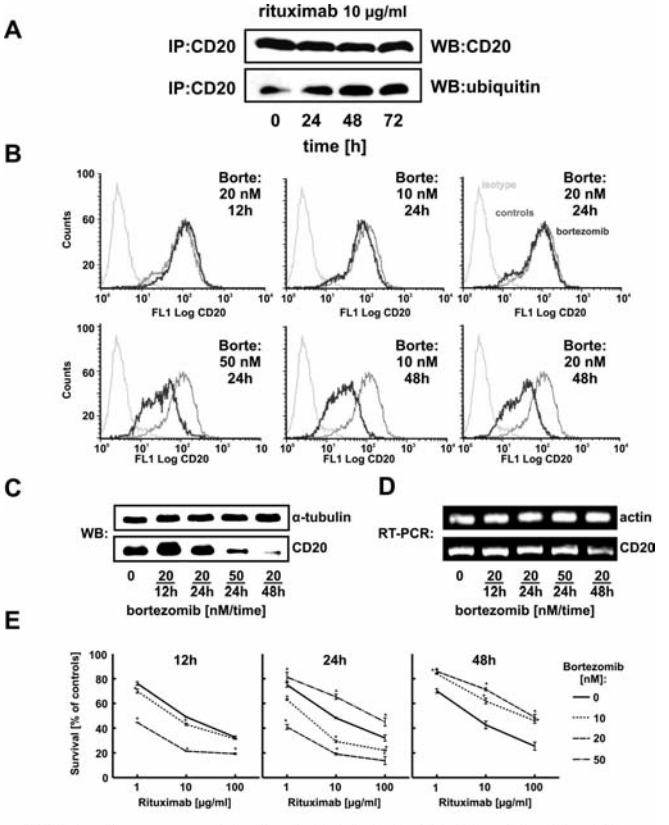
Background. The mechanism of resistance to rituximab therapy is not fully elucidated and still little is known about the molecular regulation of CD20 levels. The decreased surface CD20 levels were recently demonstrated in rituximab-resistant cell lines that also exhibited upregulation of the components of the ubiquitin-proteasome system (UPS). *Aim.* We decide to investigate in more detail the role of UPS system in regulation of CD20 levels and the influence of proteasome inhibition on rituximab-mediated CDC and ADCC against CD20-positive B-cell lymphomas. *Methods.* Rituximab-mediated cytotoxicity (R-CDC) was determined with MTT assay. CD20positive B-cell lymphomas and primary cells from patients, pretreated with proteasome inhibitors, were incubated with rituximab and 10% human serum. CD20 surface levels, as well as other surface antigens, were detected with fluorochrome - conjugated antibodies in flow cytometer. Total CD20 protein levels were assayed with Western blotting, the expression of CD20 gene was determined with RT-PCR. The ubiquitination of CD20 protein was assessed with Western blotting after immunoprecipitation with anti-CD20 antibody. The ubiquitination of membrane CD20 protein was performed as described above, preceeded by biotinylation of surface proteins. Autophagy detection was performed with acridine orange staining, bafilomycin A1 was used as an inhibitor of lysosomal/autophagosomal pathway of protein degradation. *Results.* We observed that CD20 molecule is ubiquitinated in lymphoma cells and that rituximab binding increases its ubiquitination. However, inhibition of the UPS was not associated with up-regulation of surface CD20 levels although it significantly increased its ubiquitination. Inhibition of proteasome activity resulted in bi-modal regulation of CD20 levels. Short-term (24h) incubation of Raji cells with 10 or 20 nM bortezomib did not change CD20 levels, but sensitized $CD20^+$ lymphoma cells to R-CDC (50% of survival in control cells, 20% of survival in bortezomib-pretreated cells). Prolonged (48h) incubation with 20 nM bortezomib, or

incubation with 50 nM bortezomib for 24h led to a significant decrease in surface CD20 levels as well as R-CDC (50% of survival in control cells, 80% of survival in bortezomib-pretreated cells). Bortezomib-mediated modulation of CD20 levels was also observed in primary tumor cells. The unexpected finding of decreased levels of CD20 and increased levels of its ubiquitination in bortezomib-treated cells indicates that ubiquitination might also label proteins for degradation by another proteolytic system. Indeed, we have observed induction of autophagy upon treatment with bortezomib. Also, bafilomycin A1 partly restored CD20 levels in bortezomib-treated cells. *Summary/conclusions.* Our results for the first time report that CD20 is ubiquitinated in tumor cells. These studies indicate that CD20 levels are regulated by two proteolytic systems and that the use of proteasome inhibitors might be associated with unexpected negative influence on R-CDC.



(A) Raji cells were exposed to 10 µg/ml rituximab for 24h, 48h or 72h, after which protein lysates were prepared. CD20 antigen was immunoprecipitated from probes with protein G bead slurry and subjected to Western blot analysis. Blots were sequentially probed (after stripping) with anti-ubiquitin and anti-CD20 antibodies. **(B)** Raji cells were incubated with either diluent or bortezomib (10-50 nM) for 12h, 24h or 48h. Then, cells were incubated with saturating amounts of FITC-conjugated mAb against CD20 for 30 min on ice in the dark and analyzed in a FACSCalibur. **(C)** CD20 protein levels in Raji cells incubated with either diluent or 20 and 50 nM bortezomib for 12h, 24h or 48h were assayed with Western blotting. **(D)** CD20 mRNA levels in Raji cells incubated with either diluent or 20 and 50 nM bortezomib for 12h, 24h or 48h were assayed with RT-PCR. **(E)** Raji cells were incubated with either diluent or bortezomib at 10, 20 or 50 nM concentration for 12h, 24h or 48h. Then cells were incubated for 1h with serial dilutions (from 1 to 100 µg/ml) of rituximab in the presence of 10% human AB serum as a complement source. Cell viability was measured with a MTT assay. The survival of cells is presented as percentage of corresponding diluent- or bortezomib-pretreated cells without rituximab.

Figure 1.

0424

ANALYSIS OF IMMUNOGLOBULIN HEAVY CHAIN VARIABLE GENES IN BURKITT LYMPHOMA UNCOVERS AN ANTIGEN ROLE IN LYMPHOMAGENESIS

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Burkitt lymphomas are aggressive neoplams derived from germinal center B cells. Analysis of nucleotide sequences of immunoglobulin heavy chain variable (IGHV) gene can provide insight into the stage of B cell development at which clonal expansion occurs in B cell tumours. The relationship of ZAP-70 expression and IGHV mutational status in CLL and the finding of ZAP-70 expression in some Burkitt lymphomas (BL) prompted us to analyze ZAP-70 expression in BL cells and to seek for a relationship with IGHV repertoire, somatic hypermutation (SHM) status, and intraclonal diversity (ID). We analyzed 14 BL cases of which 4 were HIV-BL and 3 were pediatric-BL. Other 13 pediatric-BL are currently under analysis. Clonality was assessed by GeneScan fragment analysis of PCR products of independent IGHV subgroups (FR1 and IGHJ-consensus primers, BIOMED-2 protocol). Another PCR was performed with the leader IGHV clonal subgroup primer and IGHC or IGHJ-consensus primer (BIOMED-2 protocol). This product was further cloned into pCRII vector and introduced into competent bacterial cells. At least 10 clones were sequenced in 2 separate reactions. Sequencing analysis and alignments were performed with the use of IMGT/V-QUEST tool. ZAP-70 expression was assessed by means of flow cytometry and QRT-PCR. The majority of cases (78.6% n=11) had mutated IGHV genes (<98% germline identity (GI)). Only 1 BL case (7.1%) presented unmutated IGHV gene (100% GI), whereas 14.3% of cases (n=2) had IGHV genes with borderline/minimally mutated status (98-99.9% GI). ZAP-70 expression was found in 78.5% of cases (n=11), hence, there was no relationship between ZAP-70 expression and mutational status of the IGHV genes. Although the number of BL was small, we observed biased use of IGHV4-39 and IGHV3-30 (35.7%), as previously reported by Trautmann et al (ASH 2009). Pediatric-BL presented a low mutational load (98% mean GI) compared to other BL (94.7% mean GI). Stereotyped aminoacid changes were detected in 57.1% of the studied BL (n=8). They were identified in cases that use IGHV4-39 and IGHV3-30 genes but also in cases with different IGHV genes and even subgroups. These shared replacement mutations strongly indicate the influence of a common antigen in the SHM process of the immunoglobulins in BL. Ongoing SHM leads to ID and reflects the persistence of antigen encounter in the germinal center reaction. For ID evaluation, only confirmed mutations, mutations observed more than once in the clones of the same BL case, were considered. ID was present in 57.1% of the 14 BL cases studied (n=8). More, all HIV-BL and all ZAP-70 negative BL showed ID being reasonable to speculate that BL cells of these particular groups are continually being stimulated by an antigen. To conclude, in BL the high IGHV mutational load observed and the evidence of ID suggested that antigen selection pressure must be involved in the malignant transformation. Moreover, the biased use of certain IGHV genes and the observed stereotyped aminoacid changes pointed that particular antigens must be implicated in BL development by stimulating proliferation of B cells that express surface immunoglobulin encoded by certain IGHV genes.

0425

NON-IG MEDIATED ABERRATIONS OF FOXP1 IN HUMAN B CELL LYMPHOMA

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Background. The FOXP1 forkhead transcription factor is targeted by rare but recurrent translocations in B-NHL, particularly marginal zone lymphoma (MZL) and diffuse large B-cell lymphoma (DLBCL). The

most common is IGH-mediated t(3;14)(p14;q32) resulting in deregulation of FOXP1 transcription by regulatory sequences of IGH. Several translocations of FOXP1 involving non-IG loci have been described, but remain uncharacterized at the molecular level. *Aims.* This study aimed at genetic and molecular characterization of non-IG FOXP1 aberrations identified in 4 lymphoma cases. *Methods.* FISH with a panel of BAC probes was applied to map the affected breakpoints at 3p14/FOXP1 and 2q36, 3q11-q13 and 10q24. Expression of FOXP1 mRNA was analyzed by quantitative RT-PCR (QRT-PCR) with primers specific for exons 5-6, 7-8, 11-12, 14-15 and 17-18. Expression of FOXP1 protein was demonstrated by immunohistochemistry (IHC) with the JC12 antibody. *Results.* All 4 cases showed an aberrant expression of FOXP1 protein by IHC. Three of them displayed various 3p14 aberrations including t(2;3)(q36;p14) (C1) (MZL), inv(3)(p14;q11) (C2) (MZL) and t(3;10)(p14;q24) (C3) (CLL in Richter transformation). In one case of DLBCL (C4) a non-IG t(FOXP1) was detected by interphase FISH. In contrast to lymphomas with t(3;14) showing the 3p14 breakpoints in the 5'end of FOXP1, all cases with non-IG FOXP1 rearrangements displayed breakpoints in the 3'end of the gene. The reciprocal breakpoints were mapped within AP1S3 at 2q36 (C1), in a region at 3q11 lacking known genes (C2) and close to NFKB2 at 10q24 (C3). In C1, AP1S3 has an opposite transcriptional orientation to FOXP1, which precludes the role of its 5' promoter in deregulation of FOXP1. Also the mechanism of FOXP1 overexpression in C2 with inv(3) affecting no known gene locus at 3q11 remains unknown. To check the hypothesis that non-IG translocations of FOXP1 result in an aberrant expression of variant FOXP1 transcripts, we performed exon-specific QRT-PCR analysis of two available cases (C2 and C4). As controls, we analyzed 2 cases with t(3;14) and 6 cases of DLBCL/MZL expressing FOXP1 but lacking FOXP1 rearrangements. These studies showed that both cases with t(3;14) expressed all analyzed coding exons (6-17) while cases with non-IGH translocations of FOXP1, as well as FOXP1$^+$ DLBCL/MZL cases expressed sequences coded by exons 7-17, but not of exon 6. *Conclusions*. Our study demonstrates that the 3p14 breakpoints of non-IG FOXP1 rearrangements in lymphoma are clustered in the 3'end of the gene. Consequently, these aberrations result in expression of variant FOXP1 transcripts, likely encoding N-terminally truncated proteins, similar to potentially oncogenic smaller FOXP1 isoforms recently identified in ABC-DLBCL cell lines and primary DLBCLs. Which transcriptional regulatory elements are engaged to aberrantly express variant FOXP1 transcripts in these peculiar non-IG FOXP1 aberrations warrants further studies.

0426

NK CELL BINDING AND INDUCTION OF POTENT NK CELL-MEDIATED ADCC BY OFATUMUMAB, A NEW HUMAN CD20 MONOCLONAL ANTIBODY

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Background. Immunotherapy with the CD20 monoclonal antibody (mAb) rituximab is part of the standard treatment for B-cell malignancies such as follicular lymphoma. Antibody-dependent cytotoxicity (ADCC) is postulated to be a key *in vivo* mechanism of action of CD20 mAb, given differences in response to rituximab based on the Fc receptor (FcγRIIIa) polymorphism. The FcγRIIIa 158V/V allotype is associated with a better response to rituximab relative to the 158F/F allotype. Ofatumumab is a new human mAb targeting a membrane-proximal epitope encompassing both the small and large loop of CD20, and has been shown to induce rapid and efficient killing of tumor cells via complement-dependent cytotoxicity. *Aims.* To investigate the ability of ofatumumab and rituximab in inducing ADCC by NK cells from FcγRIIIa 158V and 158F homozygous donors. *Methods.* Following informed consent, blood was drawn from healthy volunteers homozygous for FcγRIIIa 158V (n=10) or 158F (n=10), from a pool of donors matched for age, sex and race. Using purified NK cells, Fc-mediated antibody binding and ADCC were assessed in a blinded study. Glycan profiling was performed for ofatumumab and rituximab by HPAEC-PAD analysis. MAb binding was measured by flow cytometry in a competition binding assay in which binding of FITC-labelled CD16 mAb 3G8 was blocked by the binding of ofatumumab or rituximab. ADCC was assessed by measuring Europium release from the B-cell line ARH77 target cells upon incubation with a concentration curve of ofatumumab or rituximab in the presence of NK effector cells (effector:target ratio 5:1). Statistical analysis was performed using a non-linear mixed effect model fitting sigmoidal concentration-responses curves to data from each donor for each antibody. The model provided estimates of mean EC50 for each antibody and donor group, and allowed differences between these means to be tested for statistical significance. *Results.* Monomeric ofatumumab bound more strongly to NK cells expressing FcγRIIIa 158 V/V than FcγRIIIa 158 F/F (Table). The approximately 4.4-fold difference in affinity was statistically significant (P<0.0001). A similar 4.2-fold difference between the allotypes (P<0.0001) was found for rituximab. Rituximab bound 1.4 fold less tightly to both FcγRIIIa allotypes than ofatumumab (P<0.0001; Table). Ofatumumab induced potent NK-mediated ADCC with both FcγRIIIa 158V/V- as well as 158 F/F-expressing NK cells (Table). The observed 2.7-fold difference in potency was significant (P=0.0002). Higher concentrations of rituximab compared to ofatumumab were required to induce ADCC by NK cells obtained from both types of donors. This 1.8-fold difference in ADCC potency between ofatumumab and rituximab was statistically significant (P≤0.001; Table). *Summary/Conclusions.* Expected differences in affinity for the 158V and 158F allotypes of FcγRIIIa were observed for both ofatumumab and rituximab. These differences correlated with a stronger ADCC by FcγRIIIa 158V/V compared to 158F/F-expressing NK cells. Notably, ofatumumab induced ADCC more potently than rituximab for both Fc receptor allotypes. Ofatumumab binds CD20 stably and at a distinct membrane-proximal epitope compared to rituximab. Our data suggest that these binding characteristics may positively impact ofatumumab's ability to direct killing of tumor cells via ADCC.

Table

NK cell binding, mean EC50 (95% CI), ng/mL			
Allotypes	Ofatumumab	Rituximab	p value*
FcgRIIIa 158V/V	900 (680-1220)	1380 (1000-1890)	<0.0001
FcgRIIIa 158 F/F	4020 (2910-5540)	5730 (4090-8020)	<0.0001
p value**	<0.0001	<0.0001	--
Induction of NK cell-mediated ADCC, mean EC50 (95% CI), ng/mL			
Allotypes	Ofatumumab	Rituximab	p value*
FcgRIIIa 158V/V	6.4 (4.7-8.8)	12.6 (10.7-14.9)	=0.0002
FcgRIIIa 158 F/F	17.5 (12.9-23.7)	28.8 (24.3-34.3)	=0.001
p value**	=0.0002	<0.0001	--
*Comparison between CD20 mAbs within same allotypes. **Comparisons between allotypes using the same CD20 mAb.			

0427

A MEMBRANE-ASSOCIATED MUCIN MUC1 IS OVEREXPRESSED IN ADULT T-CELL LEUKEMIA/LYMPHOMA CELLS AND CONTRIBUTES TO ANTI-APOPTOTIC PROPERTIES

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Mucins are high molecular weight glycoprotein expressed at the luminal surface of a variety of reproductive tract epithelia. MUC1 represents a type I transmembrane glycoprotein and often becomes highly overexpressed in epithelial cancer cells and a limited types of hematological malignant cells such as myeloma cells. Adult T-cell Leukemia (ATL) is an aggressive neoplasm etiologically associated with human T-cell lymphotropic virus type-1 (HTLV-1). ATL is sub-classified into 4 clinical subtypes of acute, chronic, smoldering and lymphoma and is known to have the diversity in clinico-pathological features. Since ATL cells frequently show multi-organ invasions and are resistant to anticancer therapies, we analyzed the expression profiles and the functions of MUC1 on ATL cells. We first analyzed quantitative mRNA expression of MUC1 in various hematological cell lines: 8 ATL-related, 2 T-cell leukemia, 5 B-cell lineage, and 3 myeloid leukemia. All of ATL-related cell lines had quite high levels of MUC1 mRNA compare to the other cell lines. In primary ATL cells, acute-type (n=52) and chronic-type (n=12) ATL cases had significantly higher levels of MUC1 mRNA (P<0.01) compared with PBMC's from healthy donors (n=10). Flowcytometric analysis confirmed that ATL-related cell lines and primary ATL cells had high levels of MUC1 protein. Clinical observations in epithelial cancers have demonstrated that high MUC1 expressions are indica-

tive of an aggressive stage in tumor progression and poor survival. Meanwhile, the effect of MUC1 on tumor growth has not been clearly demonstrated. We assessed prognosis between high MUC1 mRNA expression cases and low cases of acute-type ATL. Despite the tendency that high MUC1 mRNA expression cases had poor prognosis, there was no significant difference. Of note, many of ATL-related cell lines proliferate showing auto-aggregation. Among them, we focused on KK1 and KOB cells with high levels of MUC1 expression and performed knockdown experiment of MUC1 by siRNA. After 96 hours incubation, cell growth of si-MUC1 cells was repressed in comparison with si-control cells. Next, we investigated the pattern of aggregation in KK1 and KOB cells. Interestingly, si-MUC1 cells significantly reduced aggregation after 72hours incubation in comparison with si-control cells, suggesting that MUC1 expression is a key molecule for cell aggregation. We previously found that ATL cells are resistant to TRAIL though they had death-receptors and we further investigated whether MUC1 contribute to the inhibition of apoptosis. After treatment with TRAIL for 24 hours Annexin-V positive cells of si-control cells were about 20% which increased in si-MUC1 cells to 42% in the KK1 cells. Similar effects were also observed in KOB cells. In addition, when we used a more potent apoptosis inducer MG132, si-MUC1 cells also increased the percentage of annexin-V positive cells in these cells. Consequently, we found for the first time that ATL cells had unusually high levels of MUC1 as leukemia cells. si-RNA experiments unexpectedly revealed that MUC1 contributes to the cell aggregation in a certain types of ATL cells. Furthermore, MUC1 play a role in the resistance of apoptosis in ATL cells. These findings may contribute to discover unknown function of MUC1 in leukemia cells.

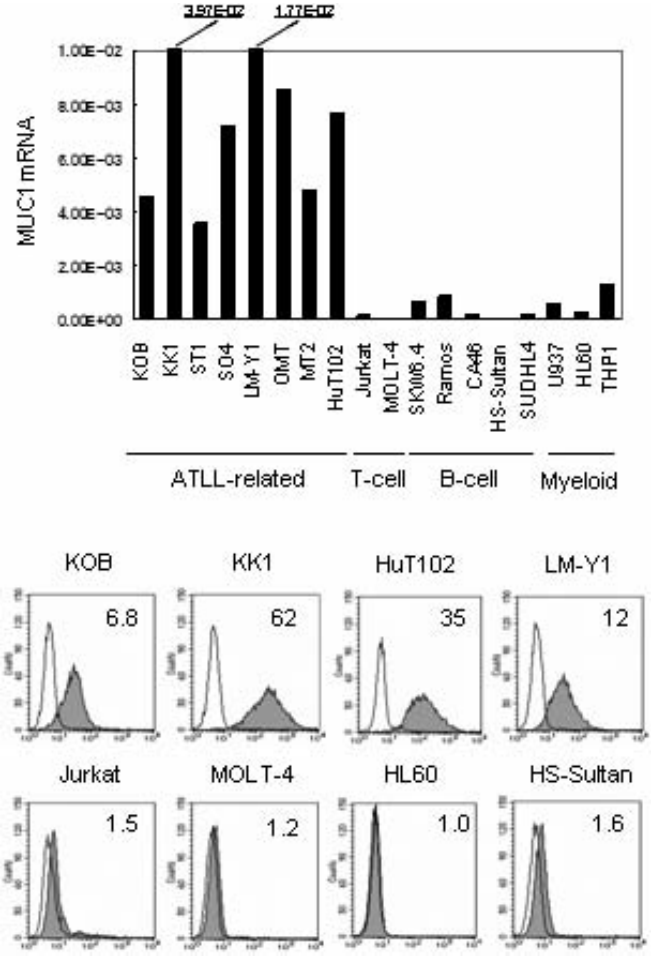

Figure. MUC1 expression in ATL cells.

0428

NON-HODGKIN LYMPHOMA SUBTYPES CLASSIFICATION USING FOCUSED MICROARRAY

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Background. Non-Hodgkin lymphomas (NHL) represent a heterogeneous group of lymphoproliferative disorders with highly variable clinical course and outcome. In some cases, current diagnostic methods based on histopathology and immunohistochemistry may be insufficient for exact tumor classification and subjectively influenced by pathologist's experience. The genome-wide transcriptional profiling was reported to accurately define the biological phenotype of the tumor. *Aims.* We have designed a novel focused oligonucleotide microarray targeted at molecular diagnostic and prognostication of lymphoproliferative disorders, particularly non-Hodgkin lymphomas. We have used the Nearest Shrunken Centroid (NSC) supervised classification algorithm (Tibshirani 2002) to test the utility of the microarray for the classification of NHLs *Methods*. The microarray based on inkjet in-situ synthesis technology contains more than 15.000 specific long-oligonucleotide probes for approximately 4.000 genes selected with emphasis on their role in lymphoproliferations as well as in crucial biological processes such as apoptosis or cell cycle control. Majority of the genes is represented by more than one distinct probe. *Results.* The classification algorithm was applied to the set of 78 NHL samples representing 3 major NHL subtypes - Follicular Lymphoma (FL), n=42, Diffuse Large B-cell Lymphoma (DLBCL), n=21 and Mantle Cell Lymphoma (MCL), n=15. For the classification the samples were randomly assigned into training and test set - 60% of samples of each NHL subtype were used to train the algorithm. The classification was performed 20 times to investigate the influence of random sampling.The NSC classification algorithm combines both gene selection and sample classification processes. For the 20 runs the best results were obtained for classifiers consisting of approximately 200 probes (median 189) translating to approximately 100 genes. The proportion of missclassified test set cases varied between 10% and 30% (median 17%), for the whole dataset the missclassification rate varied between 18% and 38% (median 27%). Most of the errors applied to wrong classification of DLBCL and FL cases, only two MCL samples were wrongly classified (in 50% and 15% of algorithm runs) both cyclin D1 negative/weak cases. *Summary*. We demonstrated successful application of gene expression based disease classification using focused microarray. The redundancy in probe coverage emerged to be an important benefit of our microarray design thus partially solving the pitfalls with probe designing such as SNPs, alternative splicing or hybridization thermodynamics. This also enhances the comparability to other microarray platforms. Our observation shows large influence of sample selection on the classification algorithm performance especially when dealing with smaller datasets. Despite the low number of cases certain NHL subtypes (MCL) can be distinguished with high confidency and even cases with aberrant phenotype could have been correctly classified.

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Reference

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0429

FCGRIIIA RECEPTOR GENOTYPE DOES NOT INFLUENCE AN OUTCOME IN PATIENTS WITH FOLLICULAR LYMPHOMA TREATED WITH RISK-ADAPTED IMMUNOCHEMOTHERAPY

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Background. Antibody (rituximab) dependent cellular cytotoxicity is a key mechanism in killing $CD20^+$ lymphoma cells. FCGR3A-158 V/F gene polymorphism results in expression of 3 variants of the FcgammaI-

IIa receptor (FCGRIIIA) on cytotoxic lymphocytes with different receptor affinity. Some studies with follicular lymphoma (FL) patients treated with immunochemotherapy demonstrated better outcomes of patients with the V/V or F/V genotypes over the F/F genotype. The role of FCGR3A-158 V/F gene polymorphism in patients treated with a risk-adapted approach has not been studied yet. *Aim.* To assess whether the FCGRIIIA receptor genotype influences the treatment response quality and outcome in patients with FL treated with risk-adapted immunochemotherapy. *Methods.* We studied 102 patients with newly diagnosed FL (grades I-IIIa) who fulfilled the GELF criteria. The median age was 52 years (31-84); 92% of the patients had advanced (III/IV) clinical stages and 56% had bulky (>7 cm) disease. The FLIPI scores were as follows: low 18.9%, intermediate 33.7% and high 47.4%. Ninety-four patients had detectable bcl-2/IgH rearrangement in bone marrow and/or peripheral blood. The front-line treatment was stratified according to the commonly used risk factors (FLIPI, beta-2-m and s-TK levels, bulky disease) into 3 treatment groups: (1) patients with FLIPI 0-1 treated with (R)-CHOP (51%), (2) patients under 60 (65) years of age with intermediate-risk disease (FLIPI 2) indicated for an intensive protocol (ProMACE-CytaBOM or sequential chemotherapy) (21%), and (3) patients under 60 (65) years with high-risk disease (FLIPI ≥3) treated with intensive chemotherapy plus autologous stem cell transplantation (BEAM) (28%). Rituximab was added to front-line chemotherapy in 59% of the patients. Genotyping of the FCGR3A-158 V/F gene was performed using PCR followed by allele-specific restriction enzyme digestion. *Results.* Generally, complete remission (CR) or unconfirmed CR was achieved in 85% of the patients, 11% had partial remission and 4% stable disease. Molecular CR (CRm) was achieved in 67.4% of 86 evaluable patients. After a median follow-up of 52 months, 57% of the patients are still in the 1st CR, 30% relapsed or progressed and 13% of the patients died. Overall survival (OS) at 5 years reached 84% (95% CI 0.74-0.93); event-free survival (EFS) at 5 years was 58% (95% CI 0.45-0.71). The frequencies of FcgammaRIIIa-158 V/V, V/F and F/F were 8%, 50% and 42%, respectively. The FLIPI score distribution was not different in F/F patients as compared to V/F+V/V carriers (chi-square, P=0.7). The treatment modalities (treatment arm or rituximab administration) had the same distribution in V/V+V/F vs F/F patients (chi-square, P=0.38 and P=0.52, respectively). The CRm rates were similar in both subgroups of V/V+V/F vs F/F patients (chi-square, P=0.92). Survival curves for OS and EFS were not significantly different when comparing the subgroups of V/V+V/F vs F/F patients (P=0.28 and P=0.57, respectively). Summary: We found no difference in the quality of treatment response or survival after front-line immunochemotherapy between FCGRIIIA subgroups. Risk-adapted intensive immunochemotherapy overcomes the negative prognostic impact of the FCGRIIIA receptor genotype. The following question is whether patients with the F/F genotype will have the same benefit from rituximab maintenance treatment. *Acknowledgements.* MSM6198959205

0430

PCR DETECTION OF NON-MTC T(11;14) TRANSLOCATIONS IN MANTLE CELL LYMPHOMA

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Background. The translocation t(11;14) (q13;q32) is the major cytogenetic hallmark of Mantle Cell Lymphoma (MCL). This translocation juxtaposes an immunoglobulin heavy chain gene (*IGH*) transcriptional enhancer on chromosome 14q32 to the protooncogene *CCND1* encoding cyclin D1 on chromosome 11q13. The translocation could be detected either by FISH or PCR. The PCR method is especially suitable for samples with low tumor load, however it is hampered by the scattering of 11q13 breakpoints. Current PCR methods are designed for detection of breakpoints clustered in Major Translocation Cluster (MTC) on 11q13 that can be found at approximately 40% of cases only. *Aims.* We have designed a set of multiplex long-range PCR assays to extend the t(11;14) detection rate at DNA level and allow the use of this tumor specific marker for monitoring of circulating lymphoma cells by real-time PCR. *Methods.* primers were designed to tile the region of approximately 400kb centromeric of *CCND1* gene at chromosome 11 and were combined with single primer designed for chromosome 14 into the set of multiplex long-range PCR assays. The first set of PCR assays was designed redundantly - in positive cases up to three different products in different PCR assays should be obtained. Second set of primers was designed to shorten the PCR amplicon and enable rapid sequencing of the breakpoint region. *Results.* For initial testing we have used 16 DNA samples obtained from lymphnode biopsies which were PCR negative for MTC breakpoints. FISH t(11;14) assay was accomplished in 7 (44%) of these samples yielding positive result in all of them.The breakpoint region was succesfully amplified in 11 (69%) and sequenced in 7 (44%) of the 16 samples. We have obtained 3 amplicons in 2 (18%), 2 amplicons in 7 (64%) and single amplicon in 2 (18%) cases. The breakpoints on chromosome 11 were scattered over the region of approx. 200kb centromeric of the *CCND1* gene. In the set of sequenced samples *IGHJ6* gene was used in 3 (43%), *IGHJ4* gene in 3 (43%) and *IGHJ3* gene in 1 (14%) case. Of the 7 FISH analysed cases amplification product was obtained in 4 (57%) of them. The breakpoint can be detected at dilution at least 1:100 as determined by serial dilution of positive DNA. *Summary.* We have demonstrated our method for multiplex long-range PCR detection of t(11;14) can increase the overall PCR detection rate to approximately 80%. It still remains lower compared to FISH assay but the PCR method could be succesfuly used even in samples with low tumor load such as periferal blood or bone marrow.

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0431

FEASIBILITY AND SAFETY REDUCING COLONY-STIMULATING FACTORS (G-CSF) IN DOSE DENSE R-CHOP THERAPY

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Background Reducing the cycle length from 3 to 2 weeks (CHOP21 vs CHOP14) in treatment of aggressive non Hodgkin lymphoma (NHL) has been demonstrated to increase OS in young patients with good prognosis, OS and EFS in elderly patients. In these studies use of G-CSF was for 10 days (from +4 to +13). Employment of G-CSF is recommended by ASCO guidelines 2006 because it allows to respect the programmed bi-weekly chemoimmunotherapy and reduce the risk of febrile neutropenia when it's equal or greater than 20%. *Aims.* In this prospective study we evaluate the feasibility of growth factors' reduction during treatment of NHL. Our aim was to demonstrate that less number of vials of G-CSF don't significantly increase the risk of haematological toxicity and infection even if we use a dose-dense chemotherapy. *Methods.* From June 2002 to July 2009 we included 89 patients with a newly diagnosed non Hodgkin lymphoma (87% diffuse large B-cell lymphoma; 10% follicular lymphoma grade IIIb; 3% other types of NHL). The median age was 62 years (range 26-75) and 32% of patients had an high-intermediate or high IPI. Patients were treated with immunochemotherapy every 14 days: rituximab on day 1 and CHOP on day 2 followed by G-CSF (lenograstim). In first ten patients we used 7 vials of G-CSF (from +5 to +11) after each cycle of chemotherapy. According to well-tolerance of treatment we prospectively decided to administer 5 vials of G-CSF(from +7 to +11). Evaluation of therapy's tolerance was made with cell blood counts at the beginning of each cycle. Further reduction of vials, at least 3 for cycle, was accepted if patients reached a number of leucocytes over 20.000/mmc. *Results.* We used a median of 25 vials of G-CSF (range 10-35) for each patient that correspond to 5 vials for each cycle (range 0-10). Due to occurrence of severe adverse events (neutropenia, piastrinopenia , infective episodes and mucositis) therapy was delayed in 21% of patients and 3 patients switched to R-CHOP21 scheduling because of poor tolerance of dose-dense chemotherapy. Eighty-six patients out 89 (96.6%) completed treatment: incidence of neutropenia grade 3-4 was 15%, trombocitopenia grade 3-4 2%, febrile episodes 10% and hospedalization in 5.6% of patients. Leucocyte nadir was 3500/mmc (400-8400), anemia nadir was 11 gr/dL (5,8-15,5) and thrombocytopenia 146.000/mmc (43000-328000). We evaluated response to therapy in 89 patients who completed the programmed scheduling R-CHOP14: complete remission rate was 87.2% with an overall response rate of 96.5%. Overall survival was 84% after a median follow-up period of 25 months (range 4-90). *Conclusion.*s Dose-dense therapy was well tolerated by modulating, according to leucocyte count, G-CSF administration and reducing the number of G-CSF's vials from 10 to 5 for cycle. The rate of neutropenia and febrile episodes were not different from those reported by literature. The be-weekly scheduling was respected in the majority of patients even if we used a less number of vials.

0432

DEPLETION OF TUMOR CELLS AND T REG CELLS AFTER TREATMENT IN GASTRIC MALT LYMPHOMA

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Background. The tumor microenvironment of follicular lymphoma and diffuse large B-cell lymphoma has been shown to play a critical role in the biology of these diseases. The clinical significance of T regulatory cells infiltrating B-cell lymphomas has been highlighted by immunohistochemistry studies demonstrating that an increased infiltration by FOXP3$^+$ cells correlates with an inferior outcome. However, the significance of the presence of T reg cells (FOXP3$^+$) in MALT lymphoma of the stomach remains unknown. *Aim.* To study by immunohistochemistry the presence of T reg cells in the microenviroment of gastric MALT (gMALT) lymphomas at the time of diagnosis and during follow-up, and to evaluate their possible impact in outcome. *Patients and Methods.* Patients were included in the study if they had gastric MALT lymphoma diagnosed according to the 2008 WHO criteria and diagnostic paraffin-embedded blocks were available for review. Thirty two patients met the criteria for inclusion in the study. As controls we used 7 gastric diffuse large B-cell lymphomas with MALT areas (gDLBCL) and 12 with chronic gastritis (5 pts H. pylori negative and 7 pts H. pylori positive). Sections were immunostained for CD20, CD3, CD4, CD8, CD68, FOXP3, PD-1, bcl-10 and Ki*67*. t(11;18) was studied by FISH and/or PCR and t(1;14) by FISH. Clonality study of the B cell receptor was done according to the BIOMED-2 protocol. The number of CD20+ tumor cells and FOXP3$^+$ infiltrating cells was quantified using a micrometric ocular (WPK 10 x mn) that has a 10 mm linear scale divided to 100 parts. *Results.* The median age was 63 y (range 32-83) with 50% being male. Stage: I in 65%, II in 25% and IV in 10%; B-symptoms in 6%. Translocation t(11;18) was present in 9 (28%) pts with gMALT; 2 additional gMALT cases had strong nuclear expression of BCL-10 without t(11;18) or t(1;14). At diagnosis, the mean (± standard deviation) number of CD20$^+$ tumor cells was different between patients with gMALT (674±234 cells/cm^2) and gDLBCL (499±221; P=0.078) or chronic gastritis (304±197; P<0.0001). The mean (± SD) number of FOXP3$^+$ infiltrating cells was different between patients with gMALT (30±30 cells/cm^2) and gDLBCL (12±8; P=0.008) but not with chronic gastritis (36±40; P=0.605). The mean number of CD20$^+$ tumor cells and FOXP3+ infiltrating cells was similar between gMALT with or without t(11;18). In five cases, treatment was not available or not given. Number of treatment analyzed for gMALT was 35, since 4 and 1 pts received treatment twice or 3 times, respectively. Treatment regimens included: eradication therapy (n=10); single or combined agent chemotherapy without rituximab (n=5); rituximab alone or CHOP-like with rituximab (n=4); fludarabine (n=8); fludarabine or bendamustine with rituximab (n=8). All but 1 case with t(11;18) was treated with eradication therapy alone. The first response evaluation was done 1-2 months after finishing treatment and showed an overall clinical response rate of 82% (CR 77%); persistence of residual disease by histology or molecular (IGHV) studies was seen in 29% and 56%, respectively. Elderly patients and those with nuclear expression of Bcl-10 responded worse (P=0.062 and P=0.004). The number of CD20$^+$ or FOXP3$^+$ cells at diagnosis did not have any influence in clinical, histological o molecular response. Overall, the mean (±SD) CD20$^+$ tumor cells and FOXP3$^+$ infiltrating cells was significantly reduced after treatment (729±210 vs 290±352; P<0.0001; 32±41 vs 18±36; P=0.016, respectively). The decrease of CD20$^+$ and FOXP3$^+$ cells was observed in the same pattern in cases with or without nuclear expression of Bcl-10.The mean number of CD20$^+$ tumor cells and FOXP3$^+$ infiltrating cells significantly decreased in responders (P<0.001 and P=0.031, respectively) but not in non responders (P=0.27 and P=0.25). Treatment with fludarabine or bendamustine with or without rituximab induced a more profound depletion in the number of CD20$^+$ cells in comparison with the other treatments (9-fold vs 2-fold reduction), but the average reduction of FOXP3$^+$cell was similar with all types of treatment (2-fold). *Conclusions.* The number of CD20$^+$ tumor cells in gMALT lymphoma is greater than in chronic gastritis or gDLBCL, while the number of FOXP3$^+$ infiltrating cells is similar between gMALT and chronic gastritis. Reduction of both CD20$^+$ cells and FOXP3$^+$ cells is seen in responding patients after all types of treatment. However, regimens with fludarabine or bendamustine with or without rituximab result in deeper reduction of CD20$^+$ tumor cells while infiltrating FOXP3$^+$ cells are moderately reduced. The value of monitoring CD20$^+$ and FOXP3$^+$ during follow-up is under analysis.

0433

ANTITUMOR EFFECT OF CELECOXIB AND A NON COX-2 INHIBITOR DERIVATIVE IN DIFFUSE LARGE B CELL LYMPHOMA

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Background. Diffuse large B-cell lymphoma (DLBCL) is the most common of the aggressive non-Hodgkin's lymphomas. Lymphoma progression depends on survival signals mediated by a functional B cell receptor (BCR). BCR activation leads to the activation of some of the main focal adhesion proteins. Celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, has demonstrated antineoplastic effect in combination with other drugs in patients with aggressive lymphomas. E7123 is a new synthetic derivative of celecoxib lacking COX-2-inhibitory function. *Aims.* To evaluate the antitumor activity of E7123, a celecoxib derivative, and its mechanism of action, including the involvement of focal adhesion proteins, in human DLBCL cell lines. *Material and Methods.* We used seven human DLBCL established cell lines. Antitumor activity was evaluated measuring cell metabolic capacity (viability) using the XTT test. To evaluate apoptotic induction by celecoxib or E7123, we performed nuclear staining with the Hoescht dye. We analyzed the alteration of the focal adhesion proteins and the activation of caspases by Western Blot. Subcellular distribution of p130Cas was analyzed by immunofluorescence. *Results.* Celecoxib and E7123 inhibit the viability of the DLBCL cell lines in a concentration-dependent manner. E7123 shows higher levels of cytotoxicity than celecoxib (IC_{50}= 13-17 μM and 44-63 μM, respectively), after 4 hours exposure. Both drugs induce the proteolysis of the focal adhesion protein p130Cas and the nuclear traslocation of its 31 KDa fragment. Moreover, E7123 inhibits the activation of other focal adhesion proteins such as FAK, PYK2, LYN and AKT. In addition, E7123 induces caspase-independent cell death associated with mitochondrial release of AIF. *Conclusions.* E7123 shows higher antitumor effect than celecoxib in DLBCL cells. This compound does not inhibit COX-2; thus, it may not induce the cardiovascular toxicity associated with the clinical use of COX-2 inhibitors. This is the first time that apoptosis through focal adhesion deregulation has been reported in DLBCL. Moreover, E7123 induces caspase-independent cell death, a mechanism not described among current DLBCL therapeutics. Based on these results, we are carrying out *in vivo* studies to continue the development of this drug as a potential new treatment in diffuse large B cell lymphoma.

0434

SPECIFIC EFFECTS EXERTED BY B-LYMPHOPROLIFERATIVE DISEASES ON PERIPHERAL TLYMPHOCYTES PROTEIN EXPRESSION

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Background. B-lymphoproliferative diseases represent a wide range of neoplastic syndromes: Hodgkin's lymphoma, aggressive and indolent lymphoma, chronic lymphocytic leukemia, plasma cell dyscrasias. Various studies describe alteration of T-cells in patients affected by such diseases (1-4) arising a great interest about the regulatory function of T-lymphocytes on B-cells pathologies. In our laboratory, we have previously demonstrated that T-lymphocytes and/or their subpopulations from peripheral blood may represent molecular sensors to be used for the evaluation of gene expression modification in physiological and pathological conditions,providing a unique and easily available biological model for integrated studies of gene expression in humans *Aims.* Here we report on the proteome profile of peripheral T-cells in patients affected by B-lymphoproliferative diseases at the onset. *Methods Subjects.* Twelve patients (4 females and 8 males, mean age 59±15) with various lymphoproliferative malignancies were selected for this study by the Haematology division of the Azienda Ospedaliera Sant'Andrea, Sapienza University of Rome, Italy. Among these, 4 were affected by diffuse_large-B-cell-lymphoma (DLBCL); 3 by follicular lymphoma (FL); 3 by classical Hodgkin's lymphoma (CHL); 2 by chronic lymphocytic leukemia (CLL). Informed consent was obtained by all patients. *Samples.* T cells were then isolated by negative selection (mean purity ≥95%) using a magnetic beads system (Pan T Cell Isolation Kit II, human, Miltenyi Biotec, Auburn, CA) and Protein separation was car-

ried out after cell lysing . Two-dimensional (2D) gel electrophoresis and MALDI-ToF mass spectrometry were used for protein separation and identification. *Interaction network analysis.* Protein-protein interaction analysis was performed using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) software version 8.1 (6). The database was queried using only experimental data as prediction method and limiting the search to no more than 100 molecular interactors, with a required minimum confidence score of 0.4 (medium level). *Hierarchical clustering of proteomic data.* Unsupervised analysis of proteomic data was performed by the GeneSpring GX 7.3 expression analysis software (Agilent Technologies). For each analysed patient, the average quantisation of spot values inside its replicate group, reported as the ratio to the average of the control replicate group, was imported as expression data. Analysis included T-cell proteome profiles of: 12 B-lymphoproliferative disorders, 10 polycystic ovary syndrome (PCO), 5 congenital adrenal hyperplasia (CAH), 2 monoclonal gammopathy of undetermined significance (MGUS), healthy subjects (single sample representative of ten pooled control subjects, as described above). Clustering was performed according to an average linkage algorithm using the following parameters: similarity measure by Pearson correlation, similar branches merged with a separation ratio of 1, minimum distance 0,001. *Results.* The analysis of the proteome profile of peripheral T-cells of patients affected by B-lymphoproliferative diseases reveals decreased levels of profilin-1 and cofilin-1 and increased levels of coronin1A and prohibitin, compared with healthy controls. The protein-protein interaction network of these proteins was studied, highlighting the actin cytoskeleton regulation as the main biological process involved in peripheral T-cells of such patients. Unsupervised cluster analysis of protein expression data shows that the recorded alteration of T-cell proteome was specifically induced by B-cells pathologies. *Discussion.* Peripheral T-lymphocytes represents easily available living biosensors, and proteins whose expression level changes significantly in specific pathological states might represent new molecular markers for follow-up testing of the disease state. All patients enrolled in this study were at the on-set of the disease, and the identified proteins differentially expressed can be regarded as markers of T-cell activation, according to many recent works reporting alterations of T-cells functionality, number and functional class balance in B-lymphoproliferative disorders (35-39). We believe it is interesting to evaluate the potential prognostic and/or diagnostic value of monitoring the expression level of these factors during the therapy.

0435

ABNORMAL T-CELL RECEPTOR REPERTOIRE PATTERN AND REGULATORY T CELL DISTRIBUTION IN PATIENTS WITH NON HODGKIN LYMPHOMA

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Background. Although most non Hodgkin lymphomas (NHL) take origin from the B-cell lineage, several studies suggest that any impairment involving the different branches of the immune system may play a role in their pathogenesis. Moreover the cross-talk among lymphoma cells and other cell types, such as for instance T-lymphocytes and antigen presenting cells, within the peritumoral microenvironment seem to deeply influence the onset and evolution of NHL. *Aims.* In order to explore the possible impact that the degree of activation of the T-cell immune system and the balance among different T-cell populations may have on the NHL pathogenesis, we analysed the T-cell receptor (TCR) repertoire and the distribution of different T-cell subsets -including regulatory T-cells (Treg)- in patients with NHL. *Methods.* Our study was based on a flow cytometric analysis performed on the peripheral blood of 8 patients (4 with indolent NHL and 4 with diffuse large B-cell lymphoma, DLBCL) and 15 age-matched controls. We first determined the frequency of $CD3^+$, $CD4^+$, $CD8^+$ and $CD16^-56^+$ T-cells. Treg were then identified by considering the $CD4^+$ cell fraction characterised by a very high (>2 log) expression of CD25 and by a very low (<2 log) expression of CD127, as well as by determining the expression of FoxP3 and CD152. TCR repertoire analysis was based on a panel of 24 beta variable (BV) family-specific antibodies, combined in groups of 3, one antibody being conjugated to FITC, another to PE, and the third to FITC/PE. Costaining was performed with anti-CD4/anti-CD8 PerCp. A BV expansion was defined as any value of BV family expression higher than the mean + 3 standard deviations calculated in normal controls. *Results.* We first showed that patients had reduced frequencies of $CD8^+$ cells (mean 21% vs 33%) and $CD16^+$ $CD56^+$ natural killer cells (11% vs 22%) when compared with normal controls, while $CD3^+$ and $CD4^+$ frequencies were similar. Patients also showed a higher frequency of Treg than controls (mean 2.68% vs 1.14%), although this increase was mainly confined to patients with DLBCL (mean 3.64%) rather than in patients with indolent NHL (mean 1.57%). Finally we determined the frequency of expanded T-cell subpopulations expressing the same TCR BV subfamilies, showing in patients and controls a similar frequency of expansions in $CD4^+$ cells (1% vs1%), besides an increased frequency of $CD8^+$ expansions in patients (4% vs 2%). When we looked at the possible influence of several disease-related factors, such as WHO lymphoma subtype, IPI score, presence of constitutional symptoms, bone marrow involvement and stage, only a diagnosis of DLBCL rather than indolent NHL was specifically associated with an increased frequency of $CD8^+$ lymphocyte expansions (5% vs 1%). *Summary/Conclusions.* Our preliminary data suggest that the T-cell branch of the immune system in patients with NHL show features which can be distinguished from those observed in normal controls. In particular, NHL patients seem to show an increased degree of activation of the TCR repertoire along with a higher frequency of Treg, which are both even more pronounced in patients with aggressive NHL.

0436

LIMITED NUMBER OF GENES DISTINGUISHES BETWEEN GERMINAL CENTRE-LIKE AND NONGERMINAL CENTRE-LIKE DIFFUSE LARGE B CELL LYMPHOMA

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Background. Diffuse large B-cell lymphoma (DLBCL) was divided into GC- (germinal centre), non-GC (non-germinal centre) and PMBL (primary mediastinal B cell lymphoma) subtypes using microarray technique. The subtypes differ in their gene expression and patients' prognosis. GC-like and nonGC-like DLBCLs were distinguished by immunohistochemistry (IHC) using CD10, Bcl6 and MUM1 proteins. IHC algorithm is less precise than gene expression and some cases remain unclassified because of borderline positivity of IHC staining. *Aims.* Our aims were to define limited number of genes significantly differentially expressed between GC- and nonGC-like DLBCLs. These genes could improve DLBCL classification. Further, we want to confirm that formalin-fixed, paraffin embedded (FFPE) tissue is material suitable for gene expression analysis. *Methods*. RNA was successfully isolated from 99% of obtained paraffin blocks. 60 patients with verified DLBCL were included. Gene expression was performed on RNA isolated from FFPE tissue and RTqPCR and $\Delta\Delta$ CT methods were used. Expression of Ki67, cyclin A2, Bcl2, MME/CD10, LMO2, CD44 a MUM1/IRF4 genes was analyzed. IHC was performed on parafin slides and DLBCL were divided into GC-like and nonGC-like groups using the algorithm by Hans et al. *Results.* 58% of included patients had nonGC-like disease. GC-like DLBCLs showed higher expression of genes LMO2 (P=0.001) and MME/CD10 (P<0.001). NonGC-like tumors showed higher expression of IRF4/MUM1 (P<0.001) and Bcl2 gene (P=0.022). In comparison to published microarray data, Ki67, CCNA2 and CD44 genes did not show different expression between GC- and nonCG-like groups when RTqPCR was performed. With obtained data discrimination function was used to generate a formula assigning new patients to GC- or nonGC-like DLBCL groups. Eight IHC borderline cases were analyzed with the formula, 5/8 (62%) were assigned as nonGC-like DLBCLs. The group of patients will be extended and clinical data will be analyzed. *Summary/Conclusions* Gene expression of MME/CD10, LMO2, Bcl2 and IRF4/MUM1 genes significantly differs between GC-like and nonGC-like DLBCL tumors. These findings can be used to improve DLBCL classification especially in IHC borderline cases. Different expression of Ki67, CCNA2 and CD44 genes found with microarray techniques was not confirmed with RTqPCR. Archive material can be successfully used for gene expression analyses. *This work was supported by Ministry of Education, Youth and Sports, Czech Republic [Grant MSMT 0021620808] and by Ministry of Health, Czech Republic [Grant NS9791-4/2008].*

0437

TARGETING INTEGRIN/CD49D/VLA-4 WITH NATALIZUMAB CAN INHIBIT ADHESION OF MALIGNANT B CELLS AND SENSITIZE THEM TO CHEMOIMMUNOTHERAPY

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Background. Compelling evidence suggests that signals from the microenvironment of the bone marrow (BM) and lymph nodes (LN) are involved in lymphoma and CLL drug resistance. The homing of malignant B cells to LN/BM leads to adhesion, mediated by molecules such as VLA-4 (CD49d) and provides pro-survival signals for malignant B cells. VLA-4 binds fibronectin and VCAM-1 and is strongly expressed on primary lymphoma cells (FL, DLBCL,MCL) and in a subset of aggressive CLL cells. Disrupting the VLA-4 dependent adhesion and signaling is a potential strategy to overcome cell adhesion-mediated drug resistance (CAM-DR). Recently, a humanized monoclonal antibody natalizumab (Biogen-Idec) targeting CD49d/VLA-4 was approved for use in Crohn's disease and multiple sclerosis, and could thus be potentially introduced into cancer therapy if proven to be effective. *Aims.* We hypothesize that natalizumab can overcome CAM-DR by disrupting VLA-4 mediated adhesion and pro-survival signaling. *Methods.* VLA-4 expressing cell lines Karpas-422, Granta-519, and MEC-1, and primary lymphoma and CLL cells were used in this study. The ability of natalizumab to disrupt cell adhesion was assessed by adding natalizumab (10 ug/mL) to adherent cells cultivated on a fibronectin coated surface. For apoptosis studies, cells were pre-incubated with various concentrations of the active metabolite of fludarabine (F-ara-A, Sigma-Aldrich), rituximab (Genentech) and natalizumab and plated on a plastic, fibronectin coated surface, or confluent HS-5 stromal cells (HS-5 cells express fibronectin and VCAM-1). The viability of cells was assessed at different time points by flow cytometry (Annexin V/PI) and/or direct cell counting of cells stained with Trypan blue (Countess Automated Cell Counter, Invitrogen). For confocal microscopy, natalizumab and rituximab were labeled with Alexa 488/568 (Alexa Fluor Antibody Labeling Kit, Invitrogen). *Results.* Natalizumab significantly inhibited the adhesion of malignant B cells to fibronectin (90% reduction in the number of adherent cells, $P<0.005$). Importantly, natalizumab itself had no effect on the viability or proliferation of cell lines or primary lymphoma/CLL cells. Adhesion to stromal cells (HS-5) provided significant protection from F-ara-A induced apoptosis ($P<0.01$). Natalizumab reduced the protective effect of stroma by ~30% ($P<0.05$) and had no effect on fludarabine treated cells cultured in non-adherent conditions (plastic surface). Interestingly, natalizumab enhanced rituximab induced apoptosis in cells cultured on plastic, suggesting that an adhesion-independent mechanism may also play a role in the synergy between rituximab and natalizumab. Moreover, natalizumab's influence on rituximab efficacy was evident in rituximab induced complement lysis assays. Similar data were obtained for several tested primary lymphoma/CLL samples. We observed co-localization of fluorescent labeled rituximab and natalizumab on the surface of malignant B cells, suggesting that natalizumab may affect rituximab binding and cell membrane distribution. *Summary*. We have demonstrated that the adhesion mediated resistance of malignant B cells to fludarabine can be successfully overcome by targeting CD49d/VLA-4 with natalizumab. We have also shown that cell adhesion can mediate resistance to rituximab, and that natalizumab enhances rituximab cytotoxicity. Our results provide a strong rationale for the development of clinical trial(s) combining natalizumab with chemoimmunotherapy in the treatment of lymphomas/CLL.

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0437a

NODAL AND NON-NODAL MANTLE CELL LYMPHOMAS DISPLAY A DIFFERENT GENOMIC PROFILE AND IGHV MUTATIONAL STATUS

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Background. Mantle cell lymphoma (MCL) bears the worst prognosis among B-cell non Hodgkin lymphomas, with a median survival of 3 years. Cases with leukemic MCL and splenomegaly without adenopathies may have a more indolent course when compared to patients with predominantly nodal disease. This difference is associated with a higher rate of mutated immunoglobulin heavy chain variable region (IGHV) genes and a less frequent expression of CD38. *Aims*. We investigated the gene expression profile of a cohort of leukemic nodal and non-nodal MCL to gain insights into the biologic features of these subgroups. *Methods*. Sixteen peripheral blood samples with >75% of clonal B-lymphocytes from untreated patients affected by MCL were evaluated by HGU133 Plus 2.0 arrays (Affymetrix). Diagnosis of MCL was supported in all cases by t(11;14)(q13;q32) and/or cyclin D1 detection. Statistical analysis, performed using the dChip software, was based on unsupervised and supervised approaches. Functional annotation and enrichment analyses were performed by DAVID and FATIGO softwares. IGHV sequencing followed ERIC recommendations. *Results.* Median age was 69 years; 10 patients (62%) were males; the median lymphocyte count was 12.3×10^9/L (4.9-140.1). Twelve cases showed splenomegaly at presentation; 9 (56%) had palpable and/or deep lymphadenopathies (nodal group), while the remaining patients had no nodes (n=7, non-nodal group). IGHV mutational status, evaluable in 15 patients, proved unmutated in all nodal MCL (one third displayed IGHV3-21 gene) and mutated in all non-nodal MCL. CD38 was positive in 8/9 nodal and 1/5 non-nodal evaluable cases. T-test selected 389 differentially expressed genes and segregated samples in 2 clusters corresponding to nodal and non-nodal groups; non-nodal MCL appeared more homogeneous than nodal cases, that grouped into 2 subclusters; one included the three IGHV3-21 cases. In the nodal group, 291 genes were up-regulated and the gene categories over-represented were: apoptosis (p-value=8.5E-03), DNA repair/response to DNA damage (p-value=7.5E-02) and, to a lesser extent, cell proliferation (P=7.9E-02). Notably, a conspicuous number of up-regulated genes were related to TP53-pathway (MDM4, TP53INP1, ZNF148). In the non-nodal group, 98 genes were up-regulated with a prevalence of the lymphocyte activation terms (P=2.8E-04). Similarly, some genes that play a role in NFKB-pathway were up-regulated: MALT1, typically altered in MALT lymphoma; TNFRSF13B that regulates B-cell homeostasis; PRDX4 and FLNA that positively regulate NFKB-pathway. It is worth mentioning that BIC was over-expressed in this series. BIC processing can generate miR155, highly expressed in Hodgkin's lymphoma, diffuse large B-cell lymphomas and chronic lymphocytic leukemia. Interestingly, the comparison between the two gene lists by FATIGO highlighted a prevalence (P=9.36E-01) of genes involved in cell adhesion (IGAE, EZR, PCDH9) in the nodal group. Given the perfect overlap between clinical presentation and IGHV sequencing, these results reflect also the differences between mutated and unmutated MCL. *Conclusions.* These findings contribute to dissect the molecular mechanisms underlying the different clinical presentation of non-nodal MCL, characterized by mutated IGHV, up-regulation of lymphocyte activation genes and down-regulation of cell adhesion genes, and offer the rationale to explore their apparent different prognosis and improve the clinical management in a broadened cohort of patients.

Novel Rx approaches

0438

LFB-R603 A THERAPEUTIC CD20 ANTIBODY MEDIATES A SUPERIOR ANTITUMORAL EFFICACY IN NON-HODGKING LYMPHOMA XENOGRAFT MODEL AND B-CELL DEPLETION IN CYNOMOLGUS MONKEYS

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Background. LFB-R603 is an anti-CD20 antibody, characterized by a specific glycosylation pattern containing a high percentage of non fucosylated antibodies molecules at the Fc site. This pattern of glycosylation increases the affinity of antibodies for human Fc ∘ RIIIa resulting in an increased antibody dependent cell-mediated cytotoxicity (ADCC) by human Fc ∘ RIIIa-expressing effector cells. This antibody is currently in phase I clinical trial in B-CLL patients and its use may be expanded to other lymphoproliferative diseases such as non hodgkins lymphoma (NHL). *Aims.* The main objective was to study the antitumoral efficacy of LFB-R603 in comparison with rituximab in a model of human follicular NHL grown as a xenotransplant in mice. In addition this study aimed to compare the ability of LFB-R603 and rituximab to deplete normal B cells in cynomolgus monkeys. *Methods.* RL cells, derived from a patient with transformed NHL were implanted subcutaneously in SCID mice. When tumors became palpable mice were randomized to receive LFB-R603 (4 weekly intravenous injections at dose levels of 10, 30 and 100 mg/kg) or rituximab (4 weekly intravenous injections at dose levels of 30 or 100 mg/kg). In a separate experiment antibodies (30 or 60 mg/kg) were administered in combination with cyclophosphamide (50 mg/kg, intraperitoneal injection) for a total of 4 injections. Tumor volumes and weight were monitored twice weekly. Depletion of non-malignant B cells by LFB-R603 was also studied in cynomolgus monkeys. Monkeys were intravenously injected once a day for 4 consecutive days at different doses: 0.012, 0.03, 0.06, 0.12 and 0.3 mg/kg for LFB-R603 and 0.06, 0.2, 0.3, 0.6 mg/kg for RTX. B-cell depletion was measured in the peripheral blood by flow cytometry. Cynomolgus B cell depletion was also evaluated during toxicological study. In this study 0.3, 10 and 100 mg/kg of LFB-R603 were administrated and B-cell depletion was also measured in lymphoid organs. *Results.* We found that LFB-R603 displayed a dose-related antitumor activity in the RL model. Furthermore LFB-R603 possessed greater antitumoral activity than rituximab in the RL model at doses of 60 and 100 mg/kg. We also observed additive cytotoxicity of cyclophosphamide and LFB-R603 against RL xenografts. In cynomolgus monkeys dose-dependent B-cell depletion was observed in peripheral blood with both antibodies. However, the total dose required for 50% depletion calculated from the dose-effect curve showed that LFB-R603 activity was almost 6 times higher than that of rituximab. On day 15 after administration of LFB-R603 at 0.3, 10 and 100 mg/kg a marked dose-dependent reduction of B-cells was observed in lymphatic tissue (axillary lymph nodes, femoral bone marrow and spleen). This effect was reversible as for the highest dose tested analysis of B-cell depletion 113 days after treatment showed the reconstitution of the B-cell population in lymphatic tissue. *Summary and Conclusions.* Compared to rituximab LFB-R603 showed a superior efficacy in a NHL xenograft model and in B-cell depletion studies performed in cynomolgus monkeys. Moreover, an antitumoral additive effect was observed with cyclophosphamide. These results suggest that LFB-R603 possesses anti-NHL activity *in vivo*, can be combined with cyclophosphamide and may be superior to rituximab.

0439

ELIGLUSTAT TARTRATE, AN INVESTIGATIONAL ORAL THERAPY FOR GAUCHER DISEASE TYPE 1: PHASE 2 RESULTS AFTER 2 YEARS

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Background. Gaucher disease type 1 (GD1), an inherited lysosomal storage disorder, is characterized by a deficiency of acid β-glucosidase and accumulation of glucosylceramide in lysosomes causing organomegaly, thrombocytopenia, anemia, and bone disease. Eliglustat tartrate (formerly Genz-112638) is a novel, oral, small molecule inhibitor of glucosylceramide synthase under development for the treatment of GD1. *Aim.* To describe efficacy and safety observations in GD1 patients after 2 years of treatment with eliglustat tartrate. *Methods.* This is an ongoing, open-label, uncontrolled, multicenter, Phase 2 clinical trial of eliglustat tartrate (50 or 100 mg bid, depending on plasma level of drug) in 26 previously untreated adults with GD1. All patients had to have splenomegaly with thrombocytopenia and/or anemia. Efficacy results included changes from baseline in spleen and liver volumes, hemoglobin and platelet levels, bone mineral density, and other skeletal findings. Hematologic and visceral parameters were assessed every 3 to 6 months; MRI, DXA, and X-rays were performed yearly and reviewed centrally. Achievement of Gaucher disease therapeutic goals for anemia, thrombocytopenia, and organomegaly also was assessed at 2 years. *Results.* Two-year treatment results (mean changes from baseline±SD) are available in up to 20 patients. Hemoglobin level increased by 2.1±1.5 g/dL (11.2±1.6 to 13.3±1.5 g/dL), and platelet count increased by 81.5±56.0%, from 67,900±20,900/mm3 to 119,200±42,400/mm3. Spleen volume (as multiples of normal, MN) decreased by 52.4±10.7%, and liver volume (MN) decreased by 23.9±12.8%. Through 2 years, no bone crises or reductions in mobility were reported. Femur MRI showed improved dark marrow signal in 8 patients and stable findings in the remaining 10 patients with available data. There were no new lytic lesions or bone infarcts. At 2 years, all 9 existing lytic lesions remained stable; of 7 existing infarcts, 1 was improved and 6 remained stable. Mean lumbar spine bone mineral density improved throughout the study period. At 2 years, there was a mean improvement in DXA Z-score of 0.60±0.69 (P=0.0033; n=16) over baseline (-1.34±1.02) and a similar mean improvement in DXA T-score of 0.56±0.78 (P=0.0115; n=16) over baseline (-1.69±1.07). Data for achievement of therapeutic goals will also be presented. Eliglustat tartrate was well tolerated in this trial up to 2 years. Most adverse events (AEs) were mild and unrelated to treatment. The most common AEs reported during 2 years were viral infections (6 patients), and urinary tract infections, increased blood pressure, and abdominal pain (3 patients each). Eight drug-related AEs, all mild, occurred in 6 patients. *Summary/Conclusions.* Eliglustat tartrate has shown promising efficacy as an oral therapy for GD1. After 2 years, patients with GD1 treated with eliglustat tartrate continued to show improvements in hematologic, visceral, and bone parameters with a safety profile that supports ongoing treatment. Two controlled Phase 3 studies"'1 in untreated patients and another in treated and stabilized patients"'are currently underway.

0440

HDAC INHIBITORS SYNERGISTICALLY ENHANCE APO866 ACTIVITY IN HUMAN LEUKEMIA CELLS: EVIDENCE FOR CONVERGING MECHANISMS OF SIRT1 INHIBITION

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Background. Cancer cells invariably exhibit aberrant histone deacetylase (HDAC) activity leading to changes in chromatine structure, altered gene expression, poor differentiation, impaired apoptosis and increased proliferation. Accordingly, virtually all the HDAC inhibitors currently available show some degree of antitumor activity in preclinical cancer models and several of these compounds are currently under investigation or already approved for the treatment of human malignancies. Furthermore Nampt (nicotinamide phosphoribosyltransferase) inhibitors such as APO866 deplete intracellular NAD^+ stores and hold promise in the treatment of hematological malignancies. Sirtuins are a large family of deacetylases characterized by a unique, NAD^+-dependent enzymatic mechanism. Recent evidence points to an emerging role for sirtuins in carcinogenesis. We have explored the combination of traditional HDAC inhibitors with the sirtuin inhibitors in leukemic cells. *Methods.* Different leukemia cell lines (Jurkat, U936 and 697) were used. Primary leukemic cells were obtained from patients with B-CLL (n=40) and AML (n=10). Cell viability was assessed by propidium iodide staining and flow cytometry. Cell signaling and apoptotic pathways were determined by Western Blot. Mitochondrial transmembrane potential (m) and intracellular NAD^+ contents were determined by tetramethylrhodamine and HPLC, respectively. The intracellular detection of Bax lev-

els was analyzed by flow cytometriy. The role of caspase activity in the cytotoxic activity was assessed using the caspase inhibitors Z-VAD-FMK (pan-caspase inhibitor). Retroviral transgenesis was performed to engineer leukemia cells to stably express a dominant negative catalytically inactive SIRT1 isoform (H363Y) or the respective empty vector. Determination of the synergistic effect of drugs combination was calculated using the CalcuSyn software based on the Chou-Talalay method. *Results.* In leukemia cells, Nampt inhibitor APO866, which reduces intracellular NAD^+ levels, synergistically increased HDAC inhibitors mediated cytotoxicity. Among the NAD^+-dependent enzymes, we subsequently identified the sirtuin family of deacetylases as involved in this interaction, since sirtuin inhibitors, but not PARP inhibitors or CD38 genetic ablation, recreate the observed synergistic lethality. As a biochemical substrate for this interaction, we found that HDAC inhibitors downregulate SIRT1 at the posttranscriptional level, thereby increasing leukemia cell susceptibility to sirtuin inhibitors. Indeed, in the presence of the dominant negative SIRT1 isoform, leukaemia cells were more efficiently killed by APO866, and sirtuin inhibitors, confirming that pre-existing SIRT1 downregulation is a condition whereupon the activity of the sirtuin inhibitors is enhanced. Moreover, HDAC inhibitors lead to Bax upregulation and cooperated with sirtuin inhibitors to promote m dissipation. Remarkably, in healthy cells, including peripheral blood mononuclear cells, normal $CD34^+$ progenitor cells and fibroblasts, HDAC inhibitors and sirtuin inhibitors were poorly active and failed to show any cooperation, suggesting a cancer-specific mode of action. *Conclusions.* Our data indicate a specific requirement by leukemia cells for sustained sirtuin activity when classical HDACs are inhibited. In the light of these results, combined HDAC and sirtuin inhibition could potentially be advised as a therapeutic strategy for human leukemias.

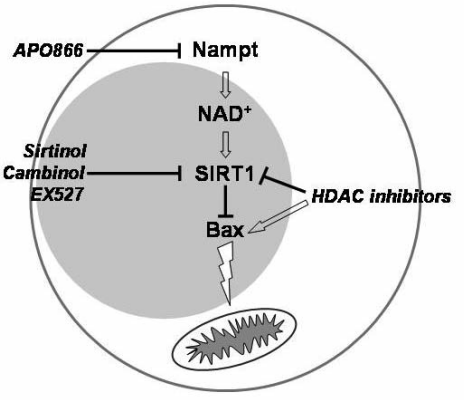

Figure. Mechanisms for the synergistic interaction.

0441

THE ANTITUMOR ACTIVITY OF MLN9708 IN GENETICALLY ENGINEERED MOUSE MODELS OF PLASMA CELL MALIGNANCY

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Background. The proteasome inhibitor VELCADE® (bortezomib) is a critical component of the chemotherapeutic strategy in treating multiple myeloma, a plasma cell malignancy (PCM). While genetically engineered mouse models (GEMMs) of cancer often accurately recapitulate their human disease counterparts, their usage in drug discovery settings have been very limited. *Aim.* Here we described the antitumor activity of a second generation proteasome inhibitor MLN9708 in the iMycCα/Bcl-XL GEMMs of PCM, in which neoplastic plasma cell development is driven by enforced expression of the Myc and Bcl-XL transgenes. *Methods.* MLN9708 immediately hydrolyzes to MLN2238, the biologically active form, upon exposure to aqueous solutions or plasma; MLN2238 was used for all preclinical studies below. We have previously demonstrated that double transgenic $iMyc^{C\alpha}$/Bcl-X_L (C57BL6/FVB) mice develop *de novo* PCM with short onset (135 days) and full penetrance (100%). We have derived a plasma cell tumor (PCT) cell line, called DP54, from the bone marrow of a syngeneic mouse previously inoculated with an $iMyc^{C\alpha}$/Bcl-X_L tumor. DP54 PCT cells were stably transfected with the firefly luciferase gene, clonally isolated, and designated as DP54-Luc cells. Nine-week-old $iMyc^{C\alpha}$/Bcl-X_L (C57BL6/FVB) mice were untreated or treated with bortezomib (1.2 mg/kg intravenously [IV] twice weekly [BIW]) or MLN2238 (18 mg/kg IV BIW) for 6 consecutive weeks and monitored for tumor-free survival for an additional 25 weeks. To establish disseminated and intraosseous mouse models of $iMyc^{C\alpha}$/Bcl-X_L PCM, freshly dissociated DP54-Luc cells were aseptically injected into the lateral tail veins and the bone marrow space of the upper right tibia, respectively, of immunocompromised mice. Once tumor growth was established, mice were randomized and treated with vehicle (5%HPbCD), bortezomib (0.8 mg/kg IV BIW) or MLN2238 (13 mg/kg IV BIW) for 3-4 consecutive weeks. The doses used represent the maximum tolerated dose for each drug treatment in each mouse strain. *Results.* In the $iMyc^{C\alpha}$/Bcl-X_L GEMM of *de novo* PCM, treatment with bortezomib and MLN2238 significantly prolonged tumor-free survival (+27 and +36 days, respectively; $P<0.0001$) and decreased plasma immunoglobulin levels compared to untreated controls. In the disseminated and intraosseous mouse models of $iMyc^{C\alpha}$/Bcl-X_L PCM, treatment with bortezomib and MLN2238 significantly reduced disease burden as measured by IVIS® bioluminescent imaging. *Summary/Conclusions.* GEMMs of cancer have often been viewed as promising alternatives to traditional subcutaneous xenograft models, yet data in which to support their wider use in drug discovery settings are sparse. Here we demonstrated that carefully implemented GEMM studies can be integrated as an important part of the drug-discovery paradigm. MLN9708 is currently in human clinical development for both hematologic and solid tumor indications.

0442

ACTIVATION OF TOLL-LIKE RECEPTORS WITH THE ANTI-MALARIAL MEFLOQUINE INDUCES CELL DEATH IN LEUKEMIA CELLS

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Background. Off patent drugs with previously unrecognized anti-leukemia activity can be rapidly repurposed for this new indication, given their prior safety and toxicity testing. *Aims.* We identified mefloquine, a quinoline licensed in oral formulation for the treatment and prophylaxis of malaria, as having potential anti-cancer activity, and sought to characterize its anti-leukemia properties. Methods and *Results.* We demonstrated that mefloquine decreased the viability of 9/9 leukemia cell lines with LD50~5.0 uM. Mefloquine reduced the viability of 6/6 primary AML blast samples, isolated from the peripheral blood of AML patients, with LD50 <5 uM, concentrations that appear pharmacologically achievable in patients. In contrast, it was significantly less cytotoxic to normal hematopoietic cells (LD50 > 40 uM). Given its *in vitro* activity, we evaluated the effects of oral mefloquine in mouse xenograft models of leukemia. Sublethally irradiated SCID mice were injected subcutaneously with OCI-AML2 or K562 human leukemia cells or MDAY-D2 murine leukemia cells, and treated with 50 mg/kg mefloquine, or vehicle alone, by oral gavage. Oral mefloquine decreased tumor weight and volume in all 3 mouse models without toxicity at pharmacologically relevant doses. To determine the mechanism by which mefloquine induces cell death in leukemia cells, we performed gene expression oligonucleotide array analysis of OCI-AML2 cells treated with mefloquine. At times preceding cell death, mefloquine altered the expression of genes associated with Toll-like receptor (TLR) and interferon response pathways. For example, we detected up-regulation of STAT1 and its downstream targets, additional TLR targets IRF1, IRF7 and IL-8, as well as NF-κB targets. Gene expression changes were validated by Q-RT-PCR, and NF-κB activity was confirmed with an ELISA-based DNA binding assay. In contrast, changes in TLR targets were not detected in normal dendritic cells resistant to mefloquine-induced cell death. Activation of TLR signaling and up-regulation of STAT1 can increase reactive oxygen species (ROS) generation. Therefore, we measured ROS generation after mefloquine treatment and demonstrated that mefloquine increased ROS production in leukemia cells, at times preceding and concentrations associated with cell death. Increased ROS production was functionally important for mefloquine-induced cell death, as blocking ROS production with N-acetyl-L-cysteine (NAC) abrogated mefloquine-induced cell death. However, NAC did not prevent up-regulation of STAT1, demonstrating that changes in STAT1 are upstream of ROS generation. To determine whether STAT1 signaling

is also functionally important for mefloquine-induced cell death, we evaluated mefloqine in 2fTGH bladder cancer cells and the U4A mutant line with a deficiency in JAK1 that is necessary for STAT1 activation. Compared to wild-type, JAK1 deficient cells were more resistant to mefloquine-induced cell death and had impaired upregulation of ROS after mefloquine treatment. Finally, we demonstrated that simultaneous knockdown of the TLR adapter proteins MyD88 and TRIF1 with siRNA abrogated mefloquine-induced cell death as well as mefloquine-induced ROS production. *Summary/Conclusions.* Our data demonstrate that mefloquine induces cell death in leukemia cells through its ability to activate multiple TLRs. Thus, this work highlights a new therapeutic strategy for leukemia. In addition, mefloquine could be rapidly advanced into clinical trial for patients with refractory leukemia.

0443

COMPARATIVE STUDIES BETWEEN ERYTHROPOIETIN (EPO) AND PBI-1402, A NEW ERYTHROPOIESIS-STIMULATING AGENT (ESA), ON MURINE CANCER MODELS

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Background. PBI-1402 is a first-in-class novel orally active compound which reduces the need for transfusion and increases hemoglobin (Hb) level and red blood cell count (RBC) in chemotherapy-induced anemia (CIA) patients. PBI-1402 promotes the production of erythrocytes by a mechanism of action which is distinct from erythropoietin (EPO). This mechanism involves differentiation of earlier progenitor stem cells (CFU-GEMM) than those affected by EPO (BFU-E, CFU-E). *Aim.* The objective of this study was to compare the effect of PBI-1402 and EPO on modulation of tumor growth. *Methods.* The effect of administration of PBI-1402 (200 mg/kg, oral, once a day) or EPO (200 or 2000 U/kg, s.c. injection, twice a week) was studied in a subcutaneous syngeneic murine P815 mastocytoma model. Tumor growth, metastasis, serum nitric oxide (NO) and hematocrit (Ht) were assessed. The effect of PBI-1402 (200 mg/kg, oral, once a day) and EPO (200 U/kg, s.c.) in combination with gemcitabine (50 mg/kg, i.p., once a week) was also studied in a subcutaneous syngeneic murine Lewis Lung (LL-2) cancer model. *Results.* These tumors express both PBI-1402 and EPO receptors on their cell surface. P815 tumor growth is rapid and metastasizes to the liver. Inflammation is also associated with P815 tumor growth. Oral administration of PBI-1402 significantly reduced tumor growth (Treated/Control (T/C): 37%) and tumor invasion as demonstrated by a 33% reduction of mice with liver metastasis. In comparison, EPO had no effect (200 U/kg, equivalent dose used in CKD treatment). However, high dose EPO (2000 U/kg, equivalent dose used in human cancer treatment) exacerbated tumor growth (T/C: 135%). Both doses of EPO induced a significant ($P<0.01$) increase in the percentage of mice with liver metastasis (2X, 200 U/kg, and 3X, 2000 U/kg) compared to control. PBI-1402 treatment had no effect on NO and Ht. However, treatment with high dose EPO significantly increased ($P<0.05$) NO and Ht level. The effect of PBI-1402 or EPO (200 U/kg) alone or in combination with gemcitabine was also studied in LL-2 cancer. Mice treated with PBI-1402 or EPO or gemcitabine alone had a T/C of 94%, 121% and 72%, respectively. Mice treated with a combination of gemcitabine plus PBI-1402 displayed a significant (synergistic) reduction of tumor growth (T/C: 33%). Mice that received gemcitabine plus EPO (T/C: 68%) did not show improvement compared to gemcitabine alone. *Conclusions.* These results suggest that oral treatment of PBI-1402 may inhibit growth and metastasis of cancer cells. They also suggest synergistic activity when used as adjuvant to chemotherapy. In contrast to EPO, PBI-1402 may offer the advantage of reducing tumor growth and metastasis while preventing anemia induced by chemotherapy.

0444

THE REGENERATION PROCESS AFTER G-CSF-MOBILIZATION IN LIVER FIBROSIS IS MEDIATED BY BONE-MARROW DERIVED AND ENDOGENOUS STEM CELLS

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Recent studies have demonstrated that bone marrow (bm) cells could potentially give rise to mature hepatocytes. We have previously shown that G-CSF accelerates the recovery process in an acute liver injury model predominantly by triggering endogenous repair mechanisms and G-CSF-mobilized hematopoietic stem cells (HSCs) produce a lasting amelioration of alcoholic cirrhosis in a pilot clinical study. Given that, we investigated the contribution of bm-derived and endogenous liver stem cells (oval cells) in the regeneration process of the chronically injured liver after G-CSF mobilization by tracking the origin of cells reconstituting liver after injury. GFP mice were used as bm donors for transplantation into lethally irradiated C57Bl6 recipients. After predominant donor chimerism was determined by flow cytometry (FCM) in peripheral blood (PB) of transplanted mice, recipients were injected with 1,5ml/kg CCl4 twice a week to produce chronic liver damage. After 2 months of CCl4 treatment, G-CSF was administered intraperitoneally at 250μg/kg/day for 7 days. Control mice received no G-CSF-treatment. Lin-sca-1+c-kit+ (LSK) cells in PB were measured by FCM to determine the successful mobilization of HSCs. Hepatic fibrosis was estimated by Gomori and Masson histostains using a five scale grading system (0-IV) and by immunohistochemistry for a-sma+ cells which represent activated hepatic stellate cells producing collagen. Immunohistochemistry in liver sections for ck19, FVIII and ki67 was used to detect hepatic stem cells, to evaluate the degree of angiogenesis and to estimate the proliferation status in each condition, respectively. Double immunofluoresence for GFP/ ck8,18 and GFP/ ck19 was used to detect bm-derived mature hepatocytes (GFP^+/ $ck8,18^+$) or endogenous liver stem cells ($GFP^-/ck19^+$). Similarly, double immunofluoresence for GFP/ki-67 was used to distinguish donor-origin proliferating cells ($GFP^+/ki67^+$) from endogenous proliferating cells ($GFP^-/ki67^+$). In $CCl4^+$G-CSF-treated mice, hepatic parenchyma maintained a rather normal architecture with limited fibrosis in contrast to extensive fibrosis seen in liver sections of CCl4-treated animals. A regeneration process was evident in both groups with increased liver mitotic activity (ki-67 positivity) in the $CCl4^+$G-CSF group. In particular, G-CSF increased the fraction of bm-derived proliferating cells ($GFP^+/ki67^+$) and more significantly the fraction of endogenous regenerating cells ($GFP^-/ki67^+$). Likewise, higher numbers of both bm-derived mature hepatocytes (GFP^+/ $ck8,18^+$) as well as *in situ* hepatic stem cells ($GFP^-/ck19^+$) could be detected in G-CSF-treated than in control group. In addition, well-shaped and dense vessels were detected in liver sections of G-CSF-treated mice accompanied by increased presence of FVIII+cells in contrast to the disorganized liver microvascular network of control mice. G-CSF attenuates liver fibrosis and facilitates hepatic regeneration by both bm-derived hepatocytes and *in situ* proliferating hepatic stem cells as well as induction of angiogenesis.

0445

METHIONINE-INDUCED HYPERHOMOCYSTEINEMIA REVERTS FIBRINOLYTIC PATHWAY ABERRATIONS IN A MURINE MODEL OF ACUTE PROMYELOCYTIC LEUKEMIA

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Background. A major component in Acute Promyelocytic Leukemia (APL) coagulation abnormality is a hyperfibrinolytic state that may be related to a high expression of Annexin II, a receptor for both tPA and plasminogen. We previously confirmed that hCG-PML-RARα leukemic mice had a higher expression of Annexin II and higher tPA levels. Homociyteine can block tPA to Annexin II linkage at a concentration of 11 μM and hyperhomocysteinemia can be induced through oral methionine supplementation. *Aims.* We proposed to block tPA linkage site in Annexin II through methionine-induced hyperhomocysteinemia in a murine model of APL (hCG-PML-RARα). *Methods.* A bone marrow transplant leukemia model was used with cells from leukemic hCG-MPL-RARα transplanted to wild type littermates after 200-cGy irradiation. One group received no treatment (LEU), one was treated with oral methionine (MET) 200 mg/Kg (q4h, 3 doses) on day 21 and another one received LCKLSL peptide (PEP), which inhibits Annexin II - tPA linkage. Four hours after peptide administration or last dose of methionine, plasma was collected from inferior vena cava after sodium citrate infusion. We also cloned Annexin II into Pichia methanolica and protein expression was confirmed by western blot followed by protein purification in nickel affinity column. Two groups of wild type mice were infused with 10 μg/Kg Annexin II. Group ANX received no further treatment and group ANXMET received oral methionine 200 mg/Kg. Tissue plasminogen activator (tPA) and plasmin levels were measured by ELISA method. *Results.* The administration of both methionine and LCKLSL peptide reduced tPA levels in leukemic mice (Group LEU: 12.14%, Group MET: 10.54% and Group PEP 11.1% -

P<0.01). In animals infused with Annexin II there was a reduction in tPA levels in methionine treated mice (ANX: 11,63%, ANXMET: 11.03% - P<0.01) which was confirmed by Plasmin levels (ANX: 302 mg/Kg, ANXMET: 235.16 mg/Kg - P<0.001). *Conclusions.* Methionine-induced hiperhomocysteinemia lowers tPA to normal leves in this model of acute promyelocytic leukemia. The same effect was observed with LCKLSL peptide, which binds to Annexin II in the same site as homocysteine, confirming the pathway of inhibition. Furthermore, in Annexin II infused mice, administration of methionine also reverted tPA and plasmin levels to normal. We conclude that methionine- induced hyperhomocysteinemia may be a approach to control APL coagulopathy.

0446

IDENTIFICATION OF POLO-LIKE KINASE-1 (PLK-1) AS A NOVEL DRUG TARGET IN NEOPLASTIC MAST CELLS IN SYSTEMIC MASTOCYTOSIS

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Systemic mastocytosis (SM) is a myeloid neoplasm characterized by abnormal growth and accumulation of mast cells (MC) in internal organs. In most patients, the D816V-mutated variant of KIT that confers resistance against several kinase inhibitors, is detectable. In aggressive SM or mast cell leukemia (MCL) the response to conventional drugs is poor and the prognosis is grave. Therefore current research is attempting to identify novel drug targets in neoplastic MC. Polo-like kinase 1 (Plk-1) is a serine/threonine kinase that plays an essential role in mitosis and has recently been introduced as a new target in myeloid leukemias. We analyzed the expression and function of Plk-1 in neoplastic human MC in patients with SM (ISM, n=27; ASM, n=3, MCL, n=2). As assessed by immunostaining, primary neoplastic MC were found to display activated/phosphorylated Plk-1 (pPlk-1) in all patients examined. The human MC leukemia cell line HMC-1 was also found to exhibit pPlk-1. In addition, we found that primary neoplastic MC as well as HMC-1 cells express Plk-1 mRNA. A Plk-1-specific siRNA induced apoptosis in neoplastic MC, whereas no effect was seen with a control siRNA. As assessed by 3H-thymidine uptake, the Plk-1-targeting drug BI 2536 was found to inhibit proliferation in HMC-1 cells (IC_{50} 5-10 nM) and in canine mastocytoma C2 cells (IC_{50} 10-50 nM). The effect of BI 2536 was seen in both subclones of HMC-1, i.e. in HMC-1.1 cells harbouring KIT G560V but not KIT D816V, and in HMC-1.2 cells exhibiting KIT G560V as well as KIT D816V, with comparable IC_{50} values. Moreover, BI 2536 was found to inhibit the proliferation of primary neoplastic MC. The growth-inhibitory effects of BI 2536 on MC were found to be associated with mitotic arrest and G2-M cell cycle arrest as well as consecutive apoptosis. In consecutive experiments, we found that pPlk-1 is expressed in neoplastic MC independent of KIT D816V. We therefore also asked whether combined targeting of KIT D816V and Plk-1 would lead to synergistic drug-effects. In these experiments, BI 2536 was found to synergize with midostaurin (PKC412) in counteracting the proliferation of HMC-1 cells, C2 cells, and primary neoplastic MC. In conclusion, our data show that activated Plk-1 is expressed in neoplastic MC in SM. Targeting of Plk-1 in neoplastic MC may be an attractive pharmacologic concept in advanced SM.

0447

PIFITHRIN μ ACTIVATES THE UNFOLDED PROTEIN RESPONSE IN CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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Introduction: Deletion or mutation of p53 genes identify a poor prognosis group of patients with chronic lymphocytic leukemia (CLL). The p53 protein plays a key role in inducing apoptosis of CLL cells in response to treatment with conventional cytotoxic drugs, therefore it is important to identify novel agents which kill CLL cells by p53-independent mechanisms. We have shown that pifithrin μ (PFTμ; 2-phenylethynesulfonamide) induces selective, p53-independent apoptosis of CLL cells (Steele et al: Blood 114:1217; 2009). Although apoptosis induction by this agent apparently correlated with upregulation of the pro-apoptotic Noxa protein, the mechanism of Noxa induction was not clarified. Recent studies have shown that PFTμ is an inhibitor of the chaperone protein Hsp70 (Leu *et al*. Mol Cell 36:15; 2009), which acts to maintain cytosolic proteins in a correctly folded state. In contrast, inhibition of chaperones within the endoplasmic reticulum (ER) results in accumulation of unfolded proteins within this compartment and consequent activation of a subset of specific intracellular pathways known as the unfolded protein response (UPR). These pathways are initiated by the activation of protein kinases IRE-1α and PERK. Under normal conditions these enzymes are supressed via binding to the ER chaperone BiP. An increase in unfolded proteins within the ER sequesters BiP, thereby allowing activation of IRE-1α and subsequently of the JNK kinase. PERK is activated in an analogous manner, resulting in the phosphorylation of the transcription factor eIF2α, resulting in striking changes in the pattern of protein translation. Here we describe studies consistent with the interpretation that the PFTμ toxicity is dependent on its ability to induce the UPR via inhibition of BiP. *Aim.* To determine whether induction of the UPR by PFTμ may contribute to its ability to induce apoptosis of CLL cells. *Methods.* CLL cells were isolated and cultured *in vitro*. Molecular responses to treatment with PFTμ and/or other agents were investigated primarily by western blotting. *Results.* Treatment of CLL cells with 10-20 μM PFTμ induced phosphorylation of JNK and its target c-JUN, which are downstream of the UPR signalling protein IRE-1α. PFTμ also induced phosphorylation of eIF2α, a target of PERK. The ER chaperone BiP was elevated in response to PFTμ, reinforcing the conclusion that this agent induced the UPR. However, the cytosolic chaperones Hsp70 and Hsp40 were also elevated, consistent with an inhibition of cytosolic chaperones in addition to ER chaperones. N-acetylcysteine, a scavenger of reactive oxygen species which relieves ER stress, blocked apoptosis induction by PFTμ and also blocked induction of UPR pathways. *Conclusions.* These data suggest that PFTμ induces ER stress and the UPR signaling pathway, in addition to inhibition of cytotsolic chaperone mechanisms. This dual mode of action suggests that PFTμ may prove to be of value as a p53-independent therapeutic agent for CLL. Since eIF2α phosphorylation has been shown to augment Noxa expression (Fribley et al: J Biol Chem 281: 31440; 2006), further studies are being carried out determine whether this mechanisms represents a major route for apoptosis induction by PFTμ.

0448

ICL670 AS A THERAPEUTIC AGENT AGAINST MANTLE CELL LYMPHOMA

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Background. Mantle cell lymphoma (MCL) is recognized as a distinct form of B-cell non-Hodgkin's lymphoma characterized by the t(11;14)(q13;q32) translocation, resulting in cyclin D1 overexpression. MCL is considered one of the most aggressive lymphoid neoplasms and one of the most challenging lymphomas to treat. To date, there is no accepted standard therapy for MCL. Several approaches including conventional chemotherapy with and without stem cell transplantation have failed to produce durable remissions for most patients. Therefore, more effective novel therapies are needed. The increasing understanding of the pathobiology of this disease enables educated use of molecular target-based therapies. In this respect, iron chelators, like ICL670, have previously been shown to exhibit antiproliferative properties. However, their effect on MCL cells has never been investigated. *Aims.* Our main goal is to study the antiproliferative effect of the iron chelator ICL670 and to reveal the biochemical mechanisms accountable for its effect in MCL cell lines. *Methods.* The effect of ICL670 was examined using the MCL cell lines: HBL-2, JEKO-1 and Granta 519. The effect of ICL670 on cell survival was assessed by XTT proliferation assay. ICL670-induced apoptosis was examined using acridine orange/ethidium bromide and Annexin V-FITC staining and analyzed by fluorescent microscope and by FACS, respectively. The biochemical mechanism involved in the ICL670-induced apoptosis was analyzed using western blot and immunoprecipitation. *Results.* The growth inhi-

bition effect of ICL670 on HBL-2 cells was determined by the XTT proliferation assay. A 50% reduction (IC_{50}) in cell viability was observed following treatment with 5.74 μM ICL670. Clinical use of ICL670 indicates that this agent is safe for human use. In agreement, we showed that normal lymphocytic cells were less sensitive to ICL670 than the MCL cell lines. We also showed that ICL670 induces apoptosis which is mediated by the upregulation of caspase-3. Analysis of the biochemical mechanism involved in the apoptosis induced by ICL670 revealed that ICL670 leads to a decrease in cyclin D1 protein levels. After incubating the cells in cycloheximide we observed that ICL670 exposure results in accelerated cyclin D1 degradation. Furthermore, pre-treatment with the proteasome inhibitor, bortezomib, followed by ICL670 exposure, resulted in complete inhibition of cyclin D1 downregulation, suggesting a role of the proteasome in cycline D1 degradation. We also demonstrated down regulation of phosphor-Rb (Ser780) expression which results in increasing levels of the E2F/Rb complex. These data indicate that the activity of the cyclin D1/cdk 4 complex is reduced following exposure to ICL670. Finally, we found that the cyclin D1 downregulation and the induction of apoptosis observed following ICL670 treatment are probably dependent on the iron chelating abilities of ICL670. *Conclusions.* We have demonstrated that the iron chelator ICL670 induces caspases-3 dependent apoptosis in MCL cell lines. The effect of ICL670 is associated with cyclin D1 downregulation and increased E2F/RB complex formation. Because iron is known to play an essential role in controlling cell proliferation, the present data indicate that ICL670, by inhibiting cyclin D1, may constitute a promising adjuvant therapeutic molecule in the strategy for MCL treatment.

0449

THE FIRST-IN-CLASS SMALL MOLECULE INHIBITOR OF NEDD8-ACTIVATING ENZYME (NAE), MLN4924, INDUCES STABLE DISEASE REGRESSION IN PRECLINICAL MODELS OF ACUTE MYELOID LEUKEMIA (AML)

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Background. The majority of patients diagnosed with acute myeloid leukemia (AML) are more than 60 years of age and their long-term prognosis is dismal. Pre-existing myelodysplasia, multidrug resistance, and co-existing morbidities limit therapeutic options for many patients. Novel approaches are urgently needed to improve clinical outcomes. Protein homeostasis is an important regulator of the biology of cancer cells. The NEDD8-activating enzyme (NAE) has been identified as an essential regulator of the ubiquitin-like molecule, NEDD8, which post-translationally directs the specificity of cullin-dependent E3 ubiquitin ligases. The cullins control the timely ubiquitination and subsequent degradation of many proteins with important roles in cell cycle progression, DNA damage, stress responses, and signal transduction. We hypothesized that disrupting NEDD8-mediated control of protein turnover would inhibit proliferation and induce cell death. We tested this hypothesis by investigating the preclinical anti-leukemia activity of MLN4924, a novel first-in-class small molecule inhibitor of the NEDD8-activating enzyme. *Aims.* To determine the preclinical activity of MLN4924 in AML. To elucidate the mechanism of action of MLN4924 in AML cells. *Results.* Nanomolar concentrations of MLN4924 (mean IC_{50} = 211 nM) selectively and potently inhibited the *in vitro* growth and survival of MOLM-13, PL-21, MV4-11 and HL-60 cells and primary AML cells from patients with different clinical and molecular features. MLN4924 treatment also dramatically disrupted colony formation and led to a dose-dependent induction of apoptosis. Co-culturing AML cells with human stromal cells did not significantly impact the pro-apoptotic activity of MLN4924. This indicates that MLN4924 can overcome the survival advantage provided by stroma. Similarly, MV4-11 cells with and without stable shRNA-mediated knockdown of FLT3 expression responded equally to treatment with MLN4924. This suggests that MLN4924 may be an effective agent for patients with FLT3 ITD and/or activating mutations in FLT3, which are associated with inferior outcomes to conventional induction therapy. Inhibition of NAE activity with MLN4924 produced a time-dependent decrease in the levels of NEDDylated cullins leading to stabilization of cullin-dependent substrates (p27, CDT-1, NRF-2, and phopsho-IκBα) and activation of the DNA damage sensor, CHK1. Treatment with MLN4924 resulted in decreased NFκB DNA-binding activity and reduced expression of key NFκB targets. Treatment with MLN4924 led to a significant increase in reactive oxygen species (ROS) generation. The antioxidant N-acetyl-cysteine significantly blunted apoptosis indicating that ROS production is an important event in MLN4924-induced cell death. The *in vivo* anticancer activity of MLN4924 was evaluated by administering MLN4924 or vehicle control to mice implanted with HL-60 xenografts. MLN4924 treatment led to a dose-dependent decrease in disease burden and 10/10 animals in the groups treated with 60 and 90 mg/kg experienced stable regressions. Analysis of specimens collected from animals following administration of a single dose of MLN4924 demonstrated *in vivo* inhibition of cullin NEDDylation and accumulation of phospho-IκBα. *Conclusions.* MLN4924 is a very promising novel inhibitor of NAE that has potent preclinical activity and has advanced into a Phase I clinical trial for the treatment of AML.

0450

IMMUNOTHERAPY OF RECURRENT B-CELL MALIGNANCIES IN ADULT AND PAEDIATRIC PATIENTS WITH FBTA05, A TRIFUNCTIONAL ANTIBODY (ANTI-CD3 X ANTI-CD20) AND ALLOGENEIC DONOR LYMPHOCYTE INFUSION

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Background. Donor lymphocyte infusion (DLI) after allogeneic stem cell transplantation (allo-SCT) displayed limited use in recurrent B-cell malignancies in paediatric and adult patients. *Objective*. Here we studied whether FBTA05, a novel trifunctional bispecific antibody targeting CD20 on lymphoma cells and CD3 on T cells, could augment graft-versus-leukaemia/lymphoma (GvL) effects while reducing the risk of undesirable reactivity against normal host cells. *Method/Study-Design*. In compassionate use, 2 children (high-grade non-Hodgkin´s lymphoma (HG-NHL)) and 8 adults (four patients with HG-NHL and four patients with p53-mutated chronic lymphocytic leukaemia (CLL)) refractory to standard therapy were treated with escalating doses of FBTA05 (range 10-2,000 microgram) and DLI after allo-SCT. *Results.* With respect to the adult patients, three out of the four CLL cases showed a prompt but transient clinical and haematological response. In one patient with HG-NHL a halt in progression for almost 4 months could be observed. In one of the children an ongoing (7 month) response, characterized by a complete disappearance of the mediastinal tumour bulk could be observed. In both, children and adults, side effects (fever, chills and bone pain) were tolerable and appeared at antibody dose levels between 40 and 200 μg. Moreover, none of the responders evaluable for graft-versus-host disease (GvHD) (survival >100 days following DLI) revealed clinical signs of active GvHD although repeated applications of DLIs up to 1×10^8 T cells/kg body weight were performed. The cytokine profile was characterized by transiently increased release of IL-6, IL-8 and IL-10. Plasma concentrations of FBTA05 reflected differences in treatment, but strictly correlated with the corresponding dosing schedules. The highest plasma concentration of FBTA05 with 0.38 microgram/mL was detectable after application of 2,000 microgram FBTA05 accompanied by the rapid clearance of antibodies within few days. So far no significant correlation with tumour burden or disease activity could be established. *Summary/Conclusions.* In summary, FBTA05 induced prompt anti-tumour responses in treatment-refractory patients. Moreover, the absence of GvHD after repeated courses of DLI indicated the potency of FBTA05 to redirect the graft-related allogeneic response towards lymphoma cells. Based on these encouraging results, a clinical phase I/II dose-escalation study of FBTA05 in combination with DLI in adult patients with CD20-positive low and high grade B-cell lymphoma after allo-SCT is now initiated.

0451

NOVEL ANTI-CD20 MONOCLONAL ANTIBODY WITH HIGH ADCC ACTIVITY AGAINST LOW DENSITY CD20-EXPRESSING TARGETS

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Background. LFB-R603 is an IgG1 anti-CD20 with increased antibody depending cell cytotoxicity (ADCC) activity via improved IgG1 binding to CD16 due to its glycosylation pattern. *Aims.* This study aimed to study the ability of LFB-R603 to mediate effective killing of targets bearing very low density of CD20 target molecules at the cell surface after

engagement of a limited effector cell number. *Methods.* We investigated the relationship between enhanced ADCC induced by LFB-R603 and antigen density on target cells using either Raji tumor cell line cells, CD20-transfected JY5 cells (JY E5.1 cells) or B-CLL patient-derived B cells bearing around 500 000, 100 000 or 20 000 CD20 molecules per cell respectively. We further investigated the impact of effector cell (E) number on ADCC mediated by LFB- R603 or rituximab of the target Raji cell (T). ADCC experiments were performed at two different E/T ratios, 5/1 and 50/1. *Results.* Results showed that LFB-R603 is able to mediate high ADCC of JY E5.1 as well as patient-derived B-CLL cells while rituximab could only mediate weak ADCC of the same targets. EC50 of lysis mediated by LFB-R603 was reached at a concentration of 0.93 ng/mL for Raji cells, 3.8 ng/mL for JY E5.1 cells and 5 ng/mL for patient derived B-CLL cells. The amount of rituximab needed to reach these levels of lysis (LFB-R603 EC50 levels) was 35 ng/mL for Raji cells, 525 ng/mL for JY E5.1 cells and >5000 ng/mL for patient-derived B-CLL cells, showing that lysis mediated by rituximab is much more sensitive to antigen density on target cells than lysis mediated by LFB-R603. We showed that maximum lysis values were 20% lower at 5/1 ratio compared to 50/1 ratio for both LFB-R603 or rituximab. However, the amount of LFB-R603 needed to reach EC50 of lysis at both 5/1 and 50/1 E/T ratios was lower than that of rituximab (LFB-R603 EC50 5/1=0.16 ng/mL, 50/1=0.14 ng/mL; rituximab EC50 5/1=2.15 ng/mL, 50/1=7.27 ng/mL). *Summary and Conclusions.* Our data showed that LFB-R603 was able to mediate high ADCC of target cells expressing low CD20 density in contrast to rituximab and that LFB-R603-mediated killing at low E/T ratio was more effective than rituximab-mediated one.

0452

INTRAFISTULAR INJECTION OF AUTOLOGOUS BONE MARROW-DERIVED MESENCHYMAL STROMAL CELLS FOR THE TREATMENT OF REFRACTORY PERIANAL CROHN'S DISEASE

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Fistulas are an invalidating, often difficult to treat, complication of patients with Crohn's disease (CD), a disabling, chronic, relapsing inflammatory enteropathy caused by dysregulation of the immune tolerance towards intestinal bacteria in genetically susceptible individuals. Mesenchymal stromal cells (MSC) have been proved to be endowed with relevant immunomodulatory properties and represent a promising tool in therapeutic approaches of regenerative medicine, as well as in immune-mediated diseases. MSC administered either intravenously infused or locally injected into colon tissue surrounding mucosal lesions proved to be of benefit in an experimental murine model of CD. Moreover, local injection of autologous, adipose tissue-derived, MSC has been already employed in clinical practice to treat complex perianal fistulas of cryptoglandular origin or associated with CD, with promising results. We investigated the feasibility, safety and efficacy of local injection of autologous bone marrow (BM)-derived MSCs for refractory CD fistulas. MSCs were isolated and expanded ex vivo in the presence of platelet lysate from BM of 12 patients (7 males, median age 33 yrs, range 16-59). Patients received intrafistular injection of MSCs scheduled every 4 weeks (median 4 infusions) and were monitored at time of each injection, and 1, 3, 6, 12 months after the last treatment. The cytokine profile of MSCs and their ability to influence apoptosis of mucosal T cells obtained from involved and uninvolved colonic areas were also analyzed. MSC expansion was successful in all patients and no adverse event was recorded during and up to 12 months after treatment. Intrafistular injection of MSCs was effective in inducing sustained closure of fistulas, with the appearance of regenerative tissue along the tracks. In particular, 7 patients (70%) benefited from complete and sustained healing of fistula tracks, while three had partial response. All patients showed a significant reduction of both CD Activity Index (pre- and post-treatment median values: 294 SD 49 and 99 SD 32 at 6 months after the last infusion; P<0.001) and Perianal Disease Activity Index (pre- and post-treatment median values: 13.0 SD 2.2 and 4.5 SD 2.4 at 6 months after the last infusion; P<0.001) reaching disease remission usually after the second procedure. The immunephenotype of circulating T lymphocytes showed progressive increase of the number of $CD4^+CD25^{bright}FoxP3^+$ cells, which became significant (P<0.01) after the second procedure and remained stable up to 6 months after the last infusion. No modification of serum cytokines was observed at any time point. MSCs caused a sort of block of the rates of both apoptotic and living cells when incubated with T lymphocytes from diseased mucosa, whilst critically increased the apoptotic rate when incubated with T lymphocytes from apparently healthy mucosa. We speculated that a number of locally injected MSC might be able to circulate in blood and/or lymphatic stream and to reach lymph node germinal centres, thus influencing T cell differentiation. These observations highlight crucial points related to MSC distribution and homing, which deserve further investigations. Local injection of autologous BM-derived MSCs appeared feasible, safe and successful in treating fistulas associated with CD.

0453

GA101 IS A HIGHLY EFFICACIOUS TYPE II ANTI-CD20 MONOCLONAL ANTIBODY THAT INDUCES DIRECT CELL DEATH BUT LOW COMPLEMENT MEDIATED CYTOTOXICITY IN B-CLL WHOLE BLOOD ASSAYS

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Background. Understanding the mechanism of action *in vivo* of therapeutic MAbs is fundamental to improve their efficacy. Unconjugated antibodies currently investigated for the treatment of B-chronic lymphocytic leukemia include anti-CD52 alemtuzumab, anti-CD20 rituximab and more recently third generation glycoengineered anti-CD20 antibody GA101. *Aim*. We have set up a whole blood assay using B-CLL samples with the view of :1) having a rapid assay to test the efficacy of novel antibodies against B-CLL or normal B cells in the circulation, 2) having a tool to dissect the role of different mechanisms of target cell killing by MAbs in a context as unmanipulated as possible. *Methods.* B-CLL whole blood samples in citrate (0.1M) were incubated for various times with alemtuzumab, rituximab or GA101 and/or blocking anti-C5 antibody Eculizumab. Death of $CD19^+$ B-CLL was measured by FACS analysis. *Results.* We first demonstrated that direct cell death, complement mediated cytotoxicity (CDC) and phagocytosis induced by different MAbs were not significantly inhibited by citrate used at the standard concentration for anti-coagulant activity (0.1 M). We then compared the efficacy and mechansim of action of alemtuzumab, rituximab and GA101. Alemtuzumab efficiently lysed B-CLL targets with maximal lysis reached already at 1-4 hours with 10 μg/mL antibody (62%). Rituximab instead induced more limited cell death (21%) with maximal lysis only at 24 hours. Lysis by both alemtuzumab and rituximab was fully complement dependent since it was inhibited by at least 90% in presence of excess blocking anti-C5 antibody eculizumab. Interestingly GA101 killed B-CLL targets with more rapid kinetics than rituximab with 19.2.% vs 23.5% cell death at 4 and 24 hours, respectively, compared to 7.9% and 21.4% for rituximab. Lysis by both Rituximab and GA101 correlated directly with CD20 expression levels (R^2=0.88 and 0.85, respectively). GA101 required at least 10 times higher concentrations than rituximab to induce equivalent complement activation, and lysis of B-CLL in whole blood was due to complement for only 50-65%. Indeed GA101 induced direct cell death of purified B-CLL or B lymphoma cell lines in addition to CDC, and this cell death involved the lysosomal pathway. Both rituximab and GA101 showed in contrast the same efficiency in phagocytosis assays but phagocytosis was low in whole blood due to excess human immunogloulins.*Conclusions.* We conclude that the major activity of alemtuzumab and rituximab in the circulation is through complement. In contrast GA101 induces both CDC and direct cell death in an additive manner. The latter may explain the major efficacy of GA101 with respect to rituximab against B-CLL samples in whole blood assays.

0454

SELECTIVE INHIBITION OF THE CHYMOTRYPSIN-LIKE ACTIVITY OF IMMUNOPROTEASOME AND CONSTITUTIVE PROTEASOME REPRESENTS A VALID ANTI-TUMOR STRATEGY IN WALDENSTROM MACROGLOBULINEMIA (WM)

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Background. Selective inhibition of chymotrypsin-like (CT-L) activity of constitutive-(c20S) and immuno-(i20S) proteasome represents a successful strategy to induce anti-neoplastic effect in malignancies. We therefore studied ONX0912, a novel selective, irreversible inhibitor of CT-L activity of i20S and c20S. *Aims.* To evaluate the distribution of i20s and c20S in WM primary cells as compared to the related normal cellular counterpart. To evaluate the role of selective inhibition of CT-L activity of i20s and c20s as an anti-tumor strategy in WM. *Methods.* WM and IgM-secreting-low-grade-lymphoma-cell lines; bone-marrow (BM)-primary CD19$^+$cells and BM stromal cells (BMSC) were used. Expression of i20S and c20S subunits (beta1/beta2/beta5; LMP2/MECL1/LMP7) were detected in primary WM cells and cell lines by an ELISA-based-assay. Cytotoxicity/DNA synthesis/cell cycle/apoptosis were measured by thymidine uptake/MTT/PI staining/flow cytometry analysis/DNA fragmentation, respectively. NF-kB activity has been evaluated using a DNA-binding ELISA-based assay. Cell signaling and apoptotic pathways were determined by Western-Blot. Determination of the additive or synergistic effect of drugs combination was calculated using the Chou-Talalay-method (CalcuSyn-software). *Results.* Primary BM-derived WM cells present with higher expression of immunoproteasome as compared to the constitutive-proteasome. ONX0912 inhibited the C-L activity of both the immunoproteasome and the constitutive-proteasome, leading to induction of toxicity in primary WM cells; as well as to apoptosis in a caspase-dependent and -independent-manner, as shown by activation of c-jun-N-terminal-kinase; inhibition of NF-kB; and initiation of the unfolded-protein-response. PR-047 induced cytotoxicity and inhibited DNA synthesis in primary WM cells; IgM-secreting-low-grade-lymphoma cells; and exerted cytotoxicity even in the context of BM milieu, by inhibiting BMSCs-induced Akt- and ERK-phosphorylation in WM cells. Moreover, combination of ONX0912 and bortezomib induced synergistic cytotoxicity in WM cells, as shown by enhanced caspases-, PARP-cleavage; and NF-KB-inhibition. *Conclusion*. These findings suggest that targeting i20S and c20S CT-L activity by ONX0912 represent a valid anti-tumor therapy in WM.

0455

FONDAPARINUX VERSUS ENOXAPARIN TREATMENT IN WOMEN WITH INFERTILITY OR PREGNANCY LOSS

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Background. Unexplained miscarriage and infertility affect approximately 6% of couples trying to start a family. Immunologic and clotting disorders have been reported in many of these patients. Enoxaparin has been a preferred anticoagulant therapy due to its relative safety and effectiveness in pregnancy. However, it requires more frequent dosing compared with newer anticoagulants such as fondaparinux. *Aims.* We compared the pregnancy success rates and safety parameters of fondaparinux versus enoxaparin, combined with immunotherapy, in patients with a history of miscarriage and/or infertility and coagulation defects. *Methods.* A total of 126 pregnancies in patients with a history of miscarriage and/or infertility were retrospectively evaluated. Of these, 63 pregnancies used fondaparinux 2.5 mg daily and 63 pregnancies used enoxaparin 30 mg twice daily. The treatment groups were similar in terms of maternal age (37.1±4.3 versus 36.8±4.5 years), the number of previous miscarriages (2.2±1.8 versus 2.4±1.8 losses), and maternal immunologic and thrombophilic status. Elevated antiphospholipid antibodies were present in 41% (26/63) of patients administered fondaparinux and 43% (27/63) of patients administered enoxaparin. Elevated NK cytotoxicity (K562 killing at an effector: target ratio of 50:1) was present in 44% (28/63) of patients administered fondaparinux and 52% (33/63) of patients administered enoxaparin. Inherited thrombophilia (polymorphism of one or more of the following genes: heterozygous or homozygous factor V Leiden R506Q, prothrombin G20210A, or plasminogen activator inhibitor 4G/5G; homozygous methylene tetrahydrofolate reductase (MTHFR) C677T; or compound heterozygous MTHFR C677T/A1298C) was present in 63% (40/63) of patients administered fondaparinux and 73% (46/63) of patients administered enoxaparin. The most common immunotherapy protocol used in the two groups was IVIG [(87% (55/63) versus 81%(51/63)] and corticosteroid [57%(36/63) versus 75%(47/63]. Informed consent was obtained by all patients for off label use of relevant drugs. The study was approved by the Institutional Review Board (WIRB Study Number 1094182). Patient confidentiality was strictly maintained. *Results.* The pregnancy success rate was 67% (42/63) for patients receiving fondaparinux and 67% (42/63) for patients receiving enoxaparin. No difference was detected in birth weight (2.8±0.7 and 3.0±0.8 kg, respectively) or gestational age at delivery (37.7±2.3 and 38.1±2.8 weeks, respectively). Vaginal bleeding occurred in 14% (9/63) of fondaparinux-treated patients and 17% (11/63) of enoxaparin-treated patients (P=0.81), typically between 7 and 9 weeks of gestation. No birth defects, severe bleeding-related complications, or serious allergic reactions were observed. *Summary/Conclusions.* In women with miscarriage and /or infertility treated with a combination of immunotherapies and anticoagulants, fondaparinux is well-tolerated and enables successful pregnancy outcomes at a rate comparable with that of enoxaparin therapy. Because fondaparinux also offers more convenient once-daily dosing and, in previous studies, fewer side effects than enoxaparin, we propose that fondaparinux may offer an attractive therapeutic alternative to enoxaparin in immune-treated pregnancy. Although equivalent outcomes were observed in our analysis, a larger study is required to achieve statistical power.

0456

PRECLINICAL EVALUATION OF CPX-351 LIPOSOME INJECTION IN COMBINATION WITH CLOFARABINE AND HYPOMETHYLATING AGENTS

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Background. We have shown that delivering synergistic cytarabine (Cyt):daunorubicin (Daun) drug ratios *in vivo* using nano-scale liposomes (CPX-351 Liposome Injection) provides dramatic efficacy improvements compared to the free drug cocktail in a wide range of preclinical leukemia models. Clinically, CPX-351 is being developed with the intent to replace conventional "7+3"Cyt:Daun therapy. Given the limited success in the clinic to combine newly developed agents for hematological malignancies with 7+3 Cyt:Daun, we examined whether CPX-351 could provide an enhanced therapeutic outcome in combination with purine antimetabolites such as clofarabine and hypomethylating agents such as azacytidine using a bone marrow-engrafting acute leukemia xenograft model. *Aim.* To determine the therapeutic benefits of combining CPX-351 with recently approved hematological malignancy drugs. *Methods.* The maximum tolerated dose (MTD) of individual drugs as well as drug combinations was determined in non-tumor bearing mice using a Q2Dx3 iv dosing schedule for CPX-351 as well as free Cyt:Daun cocktail and QDx5 ip dosing of clofarabine and hypomethylating agents. Antileukemic efficacy was determined at MTD doses for the drug combinations compared to the individual components in Rag-2M mice bearing bone marrow-engrafted CCRF-CEM human leukemia with treatment starting 21 days after leukemia cell inoculation. Therapeutic activity was determined by monitoring treatment-induced increases in life span compared to saline-treated control mice. *Results.* Combining clofarabine and azacytidine with free Cyt:Daun cocktail required a 2-fold dose reduction of Cyt:Daun (from the MTD in the absence of the added agents) in order to avoid mortality and achieve tolerable weight loss. Under these conditions, the combination treatments utilizing free drug cocktail provided minimal increase in life span (ILS) compared to the individual treatment components and absolute antileukemic activity was modest (ILS values of 24% and 27% for free drug cocktail treatments combined with clofarabine and azacytidine, respectively). Although these agents also caused the need for dose reductions when combined with CPX-351, the degree of therapeutic improvement for the combination treatment was improved over the individual components and absolute anti-leukemic activities of CPX-351-containing combinations was significantly greater than those achieved for combinations utilizing the Cyt:Daun cocktail. This effect was most pronounced for CPX-351 combined with azacytidine (ILS values of 47% and 62% for combinations containing clofarabine and azacytidine, respectively). *Conclusions.* CPX-351 was designed to enhance the antitumor efficacy of cytarabine:daunorubicin combination therapy by encapsulating both agents within a drug carrier that maintains the synergistic 5:1 molar ratio for extended times after injection. This agent

may also enhance therapeutic activity when used in combination with recently approved hematological malignancy drugs such as clofarabine and hypomethylating agents compared to combinations of these new agents with conventional Cyt:Daun therapy.

0457

THE NOVEL DNA INTERCALATOR AMONAFIDE (AS1413), DISRUPTS THE CELL CYCLE BY MECHANISMS DISTINCT FROM THOSE OF TOPO II INHIBITORS DAUNORUBICIN AND ETOPOSIDE

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Background. Amonafide-L-malate (amonafide, AS1413) is a novel DNA intercalator that induces apoptosis through mechanisms involving Topoisomerase II. Previous studies have indicated that amonafide has a number of important properties that differ from those of classical TopoII poisons: 1. Amonafide is not a substrate for Pgp, MRP-1 or BCRP, resistance mechanisms that may contribute to treatment failure with classical TopoII inhibitors. 2. Amonafide is a non-quinodal napthylamide derivative, which is structurally unrelated to anthracycline or anthracenedione classes of antileukemic agents. It therefore lacks the redox and free radical reactive pharmacophores associated with the cardiotoxicity of the anthracyclines. 3. While amonafide exerts cell killing by acting on the Topoisomerase II cycle, it does not form cleavable complexes. Further work on elucidating the action of amonafide on the Topoisomerase II cell cycle is outlined here. *Results.* Cell cycle analysis was performed on the AML cell lines Thp1 and KG1. Cells were exposed to AS1413, etoposide or daunorubicin at IC_{50} doses for 24 hours (etoposide and daunorubicin) or 48 hours (amonafide). Amonafide exposure resulted in a G2/M cell cycle arrest. Further analysis of the G2/M checkpoint by microscopic analysis of metaphase spreads from cells exposed to IC_{50} levels of amonafide showed that approximately 10% of the cells were arrested in metaphase (with chromosomes clearly visible), a higher proportion than seen in untreated cells. Furthermore, there was evidence for DNA bridge formation between cells which were not fully divided, indicating inhibition in metaphase. In contrast, etoposide treatment resulted in a G2/M arrest, with no cells visible in metaphase. Daunorubicin exhibited a G1/S arrest; while this was unexpected, the mechanism of action of daunorubicin has been shown to be less cell cycle specific, so this effect may be specific for the cell lines tested here. Again, no cells were arrested in metaphase. Cell cycle regulators such as p21 were up-regulated to a greater extent after exposure to amonafide than after etoposide or daunorubicin treatment. Further investigation of pharmacodynamic markers indicative of AS1413 clinical activity will be carried out. *Conclusions.* Amonafide has shown activity in AML, a setting where anthracyclines are currently used. The findings outlined here and the other distinctive properties of amonafide, notably evasion of multi-drug resistance mechanisms, suggest that amonafide could offer a clinical profile distinct from that of classical TopoII poisons. The value of replacing an anthracycline with amonafide is currently being tested in a large randomised phase III trial comparing amonafide plus cytarabine with daunorubicin plus cytarabine in patients with secondary AML.

0458

LONG-TERM RESULTS OF 2-CHLORDEOXYADENOSINE AND CYTOSINE ARABINOSIDE COMBINATION THERAPY IN PEDIATRIC PATIENTS WITH REFRACTORY LANGERHANS CELL HISTIOCYTOSIS.

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Background Langerhans cell histiocytosis (LCH) is a rare disease which results from clonal proliferation of pathologic Langerhans cells. Overall prognosis varies from favorable to grim depending on risk organ (RO+: liver, spleen, bone marrow and lungs) involvement and early response to therapy. Standard therapy of multisystem (MS) LCH is based on vinblastin and prednisone combination. According to recent international trials, overall survival in patients with RO involvement is 69%, and in non-responders to initial treatment - less than 20%. Since 1994 2-Chlorodeoxyadenosine (2-CdA) was reported to be effective in refractory and relapsed forms of LCH. Effect in patients with risk organ involvement is limited. 2CdA is a purine analog with established activity in hairy cell leukemia and acute myeloid leukemia. *in vitro* studies demonstrated synergism of 2-CdA and cytosine arabinoside (AraC). *Aim.* The aim was to evaluate response rate, long-term results and toxicity of combination 2-CdA + AraC therapy in patients with RO+ LCH refractory to standard treatment. *Methods*. Seven patients (6 boys and 1 girl) with RO+ LCH, refractory to initial treatment, were enrolled between 2001 and 2006. Median age at disease presentation was 11,3 months (1 month - 2,4 year). Six patients were treated with LCH II protocol and 1 patient with LCH I protocol as initial therapy. After 6 weeks of therapy disease progression was documented in 5 patients and stable active disease in two. Combined 2CdA+AraC therapy was started after 6 weeks of standard therapy in 4 patients, 11 weeks - in 1 patient. Two patients received 2CdA+AraC after several courses of multi-agent chemotherapy. Therapy details are presented in Table 1. *Results.* Transient stabilization of disease (improvement of hepatosplenomegaly, lymphadenopathy and skin rash) with subsequent fulminant progression and death was seen in 2 patients. Five patients achieved non-active disease status. Four of them had complete response. One developed cirrhosis as permanent consequence of disease. This patient died of bleeding from esophageal varices. Median interval from first line treatment start to 2CdA+AraC therapy was 60 days (37-138 days). In non-survivors there was a tendency for longer delay of salvage therapy (median 87 days vs 41 days, P=.0571. At last follow-up (median 6 years, range 3.7-7.3 years) 4 patients are alive with complete resolution of disease manifestations. No reactivations and no permanent consequences were observed. One patient has viral hepatitis C. Acute toxicity included in all cases CTC grade IV neutropenia, thrombocytopenia and anemia and life-treating infections that universally required inpatient care. No long-term toxicity was observed. *Conclusions*. 2CdA+AraC combination therapy demonstrates significant activity in refractory RO+ LCH. Earlier application may give greater chance for cure. This therapy should be provided in centers able to provide high quality AML-type supportive care.

Table 1.

№	Initial treatment	Status at 6 weeks	Therapy details	Status at the end of treatment	Status at last follow-up
1	LCH I	Progression	2CdA 5 mg/m^2/day N5, AraC 200 mg/m^2/day N5 - 2 courses	Progression	Death, disease progression
2	LCH II	Progression	2CdA 7 mg/m^2/day N5, Dauno 45 mg/m^2/day N3; 2CdA 7 mg/m^2/day N5, AraC 1000 mg/m^2/day N5; 2CdA 7 mg/m^2/day N5, AraC 1000 mg/m^2/day N5 + Ida 8 mg/m^2/day N3	Complete response	Alive
3	LCH II	Progression	2CdA 8 mg/m^2/day N5, AraC 1000 mg/m^2/day N5 – 4 courses	Complete response	Alive Hepatitis C
4	LCH II	Active disease	2CdA 8 mg/m^2/day N5, AraC 1000 mg/m^2/day N5 – 3 courses	Complete response	Alive
5	LCH II	Progression	2CdA 9 mg/m^2/day N5, AraC 1000 mg/m^2/day N5 – 3 courses (+ Dauno 45 mg/m^2/day N2 in course 3)	Progression	Death, disease progression
6	LCH II	Active disease	2CdA 6 mg/m^2/day N5, AraC 1000 mg/m^2/day N5 – 3 courses	Non-active desease Hepatic cirrhosis	Death, upper GI bleeding
7	LCH II	Progression	2CdA 6 mg/m^2/day N5, AraC 1000 mg/m^2/day N5 – 3 courses	Complete response	Alive

Quality of life and health economics

0459

THE IMPACT OF A TREATMENT FREE INTERVAL ON MULTIPLE MYELOMA PATIENTS QUALITY OF LIFE: A UK CROSS-SECTIONAL OBSERVATIONAL SURVEY

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Background. Multiple myeloma patients may experience a period of remission following their first line therapy. The degree to which this period is treatment-free will be dependent on the chemotherapy regimen received and whether a maintenance therapy was administered. However, the impact of a treatment free interval on patients' health related quality of life (HRQoL) is currently unclear. *Aim.* The aims of this study were to assess if a) during the treatment free interval (TFI) patients have higher HRQoL vs. during treatment phases; b) the length of the TFI is associated with better HRQoL. *Method.* A cross-sectional postal survey was undertaken. Patient demographics, current myeloma treatment phase and treatment details were collected. HRQoL was assessed using the EORTC QLQ-C30 (cancer specific), the MY-20 (multiple myeloma specific), and the EQ-5D (generic). Participants were identified by Myeloma UK who sent the survey to patients listed on their database (n=605). The TFI data was analysed using ordinary least square regressions; an unadjusted critical alpha of 0.05 was used given the exploratory nature of the study. The EQ-5D utility and VAS scores and each functional domain of the EORTC QLQ C30 and MY20 were included as dependent variables. In order to address the aims of the study two sets of regression analyses were conducted; one with comparative treatment phases as the predictors (1st TFI vs. first line therapy, 1st TFI vs. second line therapy and 1st TFI vs. later stage) and the other with TFI length as the predictor. *Results.* The survey response rate was 67%. Out of the 402 responses received, 370 were eligible for analysis, based on the provision of current treatment stage data. Out of the 370 patients, 12 were in first line therapy, 177 in their 1st TFI, 59 in second line and 122 in later phases of myeloma treatment. Statistically significant differences were found in favour of the 1st TFI versus the first line treatment for the EQ-5D-VAS and two domains of the EORTC QLQ-C30. Being in a 1st TFI relative to the second line treatment phase or the later phases was also significantly associated with higher HRQoL as assessed by numerous domains of the EORTC QLQ-C30, the MY-20 and the EQ-5D, with some indication that this difference was greater in later treatment phases (Table 1). A longer TFI was significantly associated with better 'physical' and 'role' functioning on EORTC QLQ C30, 'future perspectives' and 'body image' domains of the MY20 and the EQ-5D derived utility value. *Conclusion*. This survey found that both being in a TFI and experiencing a longer TFI were significantly associated with higher QoL as measured by some domains of the EORTC QLQ C30, MY20 and EQ-5D. Given these findings and the limitations associated with cross sectional studies, longitudinal observational studies are needed to further explore the QoL benefit associated with a treatment free interval.

Table 1. Association between treatment phases and HRQoL.

		Treatment Free Interval Versus					
		1st Line Treat.		2nd Line Treat.		Later Phase	
		Std Beta	*p Value*	Std Beta	*p Value*	Std Beta	*p Value*
MY 20	Disease Symptoms	.023	*.666*	.038	*.490*	.078	*.161*
	Side Effects	.083	*.110*	.199	*<.001*	.201	*<.001*
	Future Perspectives	.063	*.232*	.121	*.027*	.193	*<.001*
	Body Image	.021	*.685*	.114	*.039*	.152	*.006*
EQ5D	Utility	.069	*.204*	.087	*.122*	.138	*.015*
	VAS	.150	*.004*	.108	*.044*	.278	*<.001*
QLQ C30	Physical Function	.101	*.055*	.055	*.316*	.188	*.001*
	Role Function	.106	*.042*	.110	*.043*	.195	*<.001*
	Emotional Function	.023	*.666*	.109	*.050*	.075	*.175*
	Cognitive Function	.032	*.553*	.046	*.409*	.048	*.391*
	Social Function	.053	*.311*	.133	*.015*	.198	*<.001*

0460

INVESTIGATING PRETREATMENT QUALITY OF LIFE AND SYMPTOMATOLOGY IN NEWLY DIAGNOSED HIGH-RISK PATIENTS WITH MYELODYSPLASIA. GIMEMA AND EORTC QUALITY OF LIFE GROUP

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Background. Improving quality of life (QoL) and alleviating symptoms are the major goals of treatment in patients with myelodysplasia (MDS). However, empirically-based information about these patients' QoL and experienced symptoms is lacking. There is a pressing need to gain insight into the disease burden and the health status areas that are of major concern to better inform patients and to eventually support medical decision making. *Aims.* To compare the mean pretreatment QoL and symptom scores of patients with high-risk MDS with population-based reference values and to compare the mean pretreatment QoL and symptom scores between high-risk MDS patients differing in age, gender and risk. *Methods.* Newly diagnosed patients with intermediate-2 or high-risk IPSS score are recruited in an international prospective observational study involving 15 countries and 41 centers. Patients were classified according to the WHO histology classification and a number of socio-demographic, clinical and laboratory variables were collected prior to treatment. These included: comorbidity, performance status, platelets, hemoglobin, neutrophils, white blood cell counts, and transfusion dependency. To date, 70 patients are enrolled. QoL and symptoms were measured in the hospital using the EORTC QLQ-C30. Its standardized scores range from 0-100, with higher scores representing higher levels of functioning or higher levels of symptoms. This questionnaire has undergone rigorous linguistic cross-cultural validation and was available for all patients in the appropriate language. Mean QoL and symptom scores were compared to general population reference values, adjusted for age and gender. Statistical comparisons were adjusted for multiple testing. Differences in mean scores were expressed in Cohen effect sizes (ES; with 0.2, 0.5, and 0.8 indicating small, medium, and large ES, respectively) and clinical significance (at least 10-point difference). *Results.* Median age of patients was 73 years (45% female and 55% male) and 86% was diagnosed with IPSS int-2 risk score and 14% with IPSS high risk score. Most patients were classified as WHO, RAEB-2 (69%). When compared to general population reference values, all functioning and symptom scores indicated statistically significant impairment (P<0.01). Physical and social functioning were the most impaired functional domains with ES of 0.7. These differences were also clinically meaningful with a mean difference of 11 and 13 points, respectively. Symptoms mostly impaired were fatigue (ES= 0.9), constipation (ES=0.9) and appetite loss (ES= 1.2) all showing clinical relevance (13 to 20 points difference). No major differences were found in QoL profiles and symptoms between male and female. Older patients (< 73 versus >73 years) had clinically significant worse health outcomes only as for physical functioning and constipation. Patients classified as high-risk IPSS had a clinically significant worse profile than those with int-2 risk score mainly in terms of appetite loss and fatigue (mean scores: 23 versus 44 and 40 versus 65 respectively). *Conclusions.* Patients with MDS report a seriously impaired QoL and a high symptom burden, even prior to treatment. This knowledge can alert clinicians to better identify and treat key QoL and symptoms, particularly fatigue, constipation and appetite loss. We continue accruing patients to obtain a larger sample size to further investigate and confirm these findings.

0461

HEALTH RELATED QUALITY OF LIFE IN YOUNG HAEMOPHILIA PATIENTS FROM 9 EUROPEAN COUNTRIES IN THEIR TRANSITION FROM ADOLESCENCE TO ADULTHOOD - THE HYQOL-EUROPE STUDY

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Background. Adolescents and young adults with haemophilia are concerned by maturation and aspects such as transition from adolescence to adulthood and independence and problems related to self-management of treatment and integration in job and adult life. Their health-related quality of life (HRQoL) is not only influenced by the disease and its treatment, but also by educational, vocational and relational changes associated with transition from adolescence to adulthood. *Aims.* The HyQoL-Europe - 'Helixate treated young Haemophiliacs' Quality of Life' Study was designed to provide a deeper insight into HRQoL in order to evaluate the role of transitional events and impact on haemophilia treatment. HRQoL and psychosocial determinants such as sexuality, religious beliefs, physical activity and adherence to treatment in haemophilic adolescents and adults will be assessed. Moreover, the study will identify key transitional life events that might have an impact on HRQoL such as living situation, partnership and professional situation. *Methods.* In this prospective, longitudinal, multicenter, non-interventional study 150 patients aged 14-35 years, affected by moderate or severe haemophilia A using Helixate from 9 European countries are enrolled. HRQoL is assessed by means of generic (SF-36, EQ-5D) and disease-specific (Haemo-QoL, Haem-A-QoL) questionnaires; physical functioning (HEP-Test-Q) and activity level (short EPIC Norfolk index) are evaluated with validated instruments. In addition psychosocial determinants such as sexuality, religious/spiritual beliefs, etc. are assessed with ad-hoc developed questionnaires. Clinical data are collected by physicians on bleeding history, treatment modalities, orthopaedic status, etc. by means of a modular constructed medical documentation. All evaluations will be carried out at baseline and yearly for 3 years. Differences in HRQoL will be estimated across countries or project regions and between different patient groups (e.g., adolescents vs. adults, family status, personal life circumstances, etc.). *Results.* 9 countries from 3 regions in Europe have been identified capturing different socio-economic situations and cultural aspects allowing a variety of different transitional situations (Regions: I: Germany, Austria, Switzerland, II: France, Belgium, Netherlands, III: Greece, Italy, Spain). So far, 6 patients have been enrolled from Italy. *Conclusions.* The HyQoL-Europe Study together with its sister project in Canada are the first studies looking at the crucial period of transition in the life of young haemophilia patients.

0462

DEVELOPMENT AND VALIDATION OF A HAEMOPHILIA-SPECIFIC QUESTIONNAIRE FOR THE ASSESSMENT OF HEALTH-RELATED QUALITY OF LIFE IN ELDERLY HAEMOPHILIACS (HAEM-A-QOLELDERLY)

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Rationale. Life expectancy of people with haemophilia (PWH) has increased enormously over the last 3 decades resulting in more PWHs reaching an old age. This is due to the improvement of haemophilia care, however, only few information are available on how this impacts on patients' well-being and health-related quality of life (HRQoL). For the adequate assessment of HRQoL standardised and validated instruments are necessary. Only one geriatric-specific HRQoL instrument is available for elderly people (WHOQOL-OLD), but no haemophilia-specific instrument exist for elderly haemophilia patients. In the frame of a retrospective case-control study the first haemophilia-specific HRQoL questionnaire for elderly patients was developed and validated. *Methods.* The validated disease-specific Haem-A-QoL questionnaire for the assessment of HRQoL in adult haemophilia patients was adapted for elderly patients (Haem-A-QoLElderly), since not all domains were applicable for elderly persons (such as work, family planning). Focus groups with physicians and with elderly haemophilia patients were conducted. Statements of these focus groups were content analysed and additional aspects were formulated as items and included in the existing version of the Haem-A-QoL. The pre-final version of the Haem-A-QoLElderly consisted of 106 items on a five-point Likert scale pertaining to 14 dimensions (physical health, feeling, view, family, friends, perceived support, others, sport/leisure time, work, dealing, treatment, centre, future, sexuality) and one total score. The Haem-A-QoLElderly was filled in by haemophilia patients ≥65 years and psychometrically tested for reliability (Cronbach's alpha, test-retest-reliability) and validity. Convergent validity was determined by means of the Pearson correlation coefficients comparing the Haem-A-QoLElderly scales with the generic EQ-5D, WHOQOL-BREF and WHOQOL-OLD. Discriminant validity was tested for clinical subgroups such as invalidity, orthopaedic status, viral infections and number of bleeds. *Results.* 39 haemophilia patients with a median age of 68 years (65-78) filled in the Haem-A-QoLElderly, representing 84.6% of all severely affected elderly haemophilia patients registered in Italy. 85% had haemophilia A, 21% received prophylactic treatment and 37% reported chronic pain. In average 8.6 joint bleeds were reported in the past 12 months. Patients suffered from viral infections (87% chronic hepatitis C, 13% HIV) and inhibitors (13%) and impairments were found in their orthopaedic joint status (OJS: M=19.8+15.2). Psychometric analyses showed that the newly developed Haem-A-QoLElderly has good values for internal consistency ranging from Cronbach's α=.647-.931 for the subscales and for the total score α=.964. Haem-A-QoLElderly showed good discriminant validity for invalidity, orthopaedic joint status, number of bleeds and chronic pain. The final questionnaire consists of 63 items pertaining to 11 dimensions. Elderly patients were mainly impaired in the dimension 'sport' (M=59.01+32.6), 'physical health' (M=49.72+31.0) and 'view of themselves' (M=49.30+27.3). In a linear regression model including infections, no of total bleeds, invalidity, OJS, marital status and depression 65.3% of the variance in Haem-A-QoLElderly could be explained only by depression. *Conclusions.* It could be demonstrated that the Haem-A-QoLElderly is a reliable and valid instrument for the assessment of HRQoL in elderly haemophilia patients. Since depression has the greatest impact on HRQoL compared to other predictors, special care should be provided to this patient population.

0463

ASSESSING SYMPTOMS IN HEMATOLOGY FROM THE PATIENTS' PERSPECTIVE: FEASIBILITY OF A PATIENT REPORTED INSTRUMENT IN CLINICAL RESEARCH

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Background. Patient Reported Outcome (PRO) assessments introduce the patient's perspective into the clinical research process via standardized and methodologically sound self-report questionnaires. Appropriate PRO instrument selection, however, require careful attention of a number of issues (e.g. disease stage and phase of treatment, concerns about respondent burden and feasibility and sensitivity of the measure being used). *Aims.* To investigate the feasibility and sensitivity of a validated cancer patient-reported symptom tool (MD Anderson Symptom Inventory-MDASI) in patients with hematologic malignancies. To investigate pattern of symptoms prevalence of patients in curative phase or palliative phase of treatment. *Methods.* Patients with various hematological diseases are being enrolled in a prospective study comparing a number of outcomes between those followed in a hospital-based setting versus an home-care-based program. A number of socio demographic and clinical laboratory data are collected including: comorbidity, functional independence (ADL scale), neutrophils, white and red blood cell counts and haemoglobin level. To date a total of 86 patients have been included. Patient symptoms are being measured with the MDASI which consists of 19 items (i.e. 13 items assessing symptom severity and 6 items assessing symptoms interference with various aspects of the patient's life). Items are rated on a numeric rating scale from 0 to 10, with the higher scores indicating a higher level of symptoms or a higher symptom interference. The symptom items were classified according to the following scores: mild (1-4), moderate (5-6) and severe (7-10). Descriptive statistics and linear regression analy-

ses were used. All statistical comparisons were adjusted for multiple testing. *Results.* Baseline data are available for 86 patients (26% curative phase and 74% palliative phase). Mean age was 65 years with 48% being female and 52% male. The majority of patients were diagnosed with acute myeloid leukemia (42%), non Hodgkin lymphoma (20%) and myelodysplastic syndromes (10%). Accuracy of questionnaire completion was optimal with more than 80% of patients completing all items. The percentage of missing items was low ranging between 1% to 5% (symptom interference with work activity). The top three moderate to severe symptoms in terms of prevalence in patients in palliative phase of treatment were: fatigue (80%), dry mouth (57%) and distress (54%). In patients undergoing curative treatment top three moderate to severe symptoms were: fatigue (50%), lack of appetite (46%) and nausea (38%). Details on mean symptom scores for both patient populations are reported in Figure 1 suggesting also the MDASI being able to discriminate different profiles in haematological patients based on type of treatment (curative vs. palliative). Except for lack of appetite, vomiting and nausea, all mean scores were statistically significant different at (P<0.05). Means of symptom interference for those in curative and palliative phase of treatment were 4 (SD=2.5) and 6.6 (SD=2.5) respectively, indicating symptoms severity having a higher impact on patients' life undergoing palliative treatments. *Conclusions.* The results suggest the MDASI being a feasible tool that can be successfully implemented in haematological research; it is sensitive to different populations undergoing various treatments. Regardless of patients' stage, fatigue is the most relevant symptom identified by this measure.

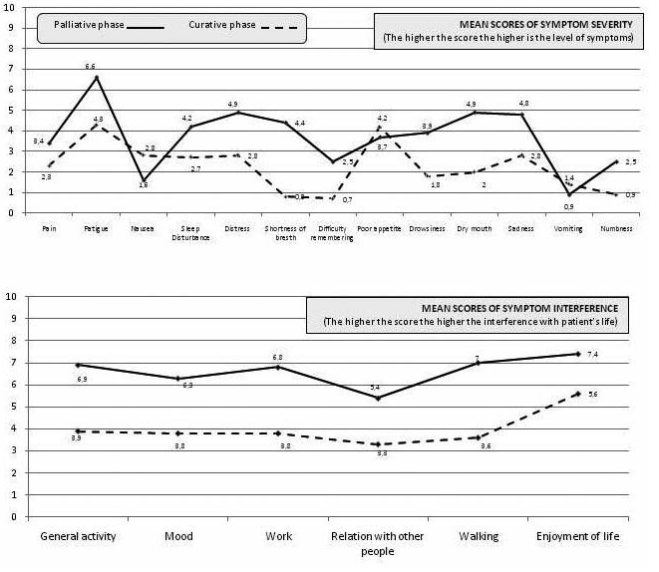

Figure 1. MDASI: symptom burden of patients in palliative or curative phase of treament.

0464

EFFECTS OF DIFFERENT TREATMENTS ON GERIATRIC ASSESSMENT AND QUALITY OF LIFE PARAMETERS IN ELDERLY MDS/AML PATIENTS

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Background Treatment allocation of elderly patients (pts) with MDS/AML to either intensive or non-intensive approaches remains difficult. In order to optimally inform the pt about different treatment options, it is essential to obtain insight into the possible development of dependence, frailty, depression and quality of life (QOL) during/after treatment. Geriatric and QOL assessment is an in-depth evaluation permitting to objectify a number of these variables. Very little is known about the development of subjective and objective parameters relevant to elderly pts under different treatment options. Aims. To study the development of parameters of a geriatric and QOL assessment in pts with MDS/AML ≥60 years over a period of 6 months under three different therapeutic approaches. *Methods.* The study cohort included all pts >/= 60 years with the initial diagnosis of MDS/AML (2004-2008) at the University Hospital Freiburg for treatment initiation. The instruments applied included those recommended for a comprehensive geriatric assessment (ADL; IADL, Comorbidities (HCT-CI), 'timed up and go test', Mini Mental State Examination (MMSE), Geriatric Depression Scale (GDS)) as well as performance status (Karnofsky Index) (PS) and items of the QOL questionnaire EORTC C-30 (global QOL, fatigue). A first assessment was performed at time of respective treatment start. To avoid attrition, only data of 119 surviving pts were evaluated for descriptive statistics, calculation of relevant deterioration/improvement in follow-up parameters and comparison between treatment groups best supportive care (BSC), hypomethylating agents (HA) and induction chemotherapy (IC). *Results.* Follow-up assessments were conducted after a median of 140 days of treatment:15.1% (n=18) BSC, 30.2% (n=36) HA and 54.6% (n=65) IC. Median age was 74.5 yrs (range 64-83), 72.5 yrs (62-82) 67 yrs (60-77) for BSC, HA and IC groups, respectively. AML (WHO) was evident in 88 (73.9%) pts. Significant differences between the following baseline parameters could be detected between treatment groups: PS BSC 73.9±13.3, HA 82.4±12, PS IC 74.9±10.6 (P=0.0093), HCT-CI BSC 3±2.3, HA 1.7±1.5 and IC 2.7±2.1 (P=0.032). At follow-up, global QOL and fatigue showed a marked improvement in 31.2% of BSC, 41.6% of HA and 39.7% of IC pts and in 50% of BSC, 44.4% of HA and 53.9% of IC pts, respectively. About 1/3 of all pts experienced a deterioration/decrease in these parameters regardless of treatment. Geriatric assessment detected new dependencies (ADL/IADL) in 11.7-32.3% of pts, while depression and cognitive changes became obvious in 4.7-24.2% of pts. PS decreased in 37.5% of BSC, 49.9% of HA and 35.4% of IC pts. *Summary/Conclusions* Geriatric and QOL assessment evokes awareness of relevant changes in elderly MDS/AML pts that might otherwise be unnoticed. Parallel to the occurrence of new dependencies and impairments that became apparent to very similar degrees in surviving pts across all treatment groups, global QOL deteriorated in 1/3 of pts. Focused interventions according to the results of repeat geriatric and QOL assessments may ameliorate impairments and dependencies.

0465

DEVELOPMENT AND FEASIBILITY OF A PATIENT-REPORTED SYMPTOM CHECKLIST FOR CHRONIC MYELOID LEUKEMIA PATIENTS

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Background. Patient Reported Outcome (PRO) instruments are now crucial to evaluate overall treatment effectiveness in oncology. While, these are broadly available for several cancer populations, there is lack of tools for patients with hematologic malignancies. Particularly, no PRO instrument exist for CML patients. *Aims.* To report early development of a brief patient-reported symptom measure to be used with CML patients undergoing treatment with Imatinib (IM) and evaluate patients' acceptance and feasibility in clinical research. *Methods.* This development followed various steps. Preliminary literature search was carried out to identify relevant issues for this patient population using several electronic databases (i.e. MEDLINE, EMBASE, CINAHL and PSYCHINFO). The list of potential issues/themes generated from the literature search was then presented to health care professionals (including clinicians and research nurses) for feedback on appropriateness of content and breadth of coverage. The list was also pilot tested on a small sample of CML patients for further refinement and issues were then operationalised into questions. Item response were designed as a 4 point Likert scale ranging from 'not at all' to 'very much' with standardized scores ranging between 0 and 100 (i.e. higher scores indicating higher symptom intensity). This checklist was then administered to a larger sample of patients in treatment with IM as part of a study investigating a number of health outcomes. Descriptive statistics were used for the sociodemographic and clinical data to characterize the sample. Distribution of missing values was investigated and Pearson's correlation coefficients between items were calculated. Discriminant validity was assessed by comparing sub-samples that were expected to differ in their symptomatology. Statistical comparisons were adjusted for multiple testing. *Results.* The literature search yielded some 300 potential articles that were independently scrutinized by two authors. Only 12 articles dealt with PRO issues in CML patients, thus additional literature dealing with IM toxicity was searched. Feedback for relevance ensuring content validity was received from 10 healthcare professionals and 11 CML patients. The list was eventually reduced to 9 key items. Analysis was then undertaken on a sample of 249 CML patients with a median age of 56 years (62% male and38% female). The large major-

ity (78%) in treatment with IM at a dose of 400 mg. Fatigue, muscle cramps and edema had the highest mean scores being 41 (SD=27), 39.6 (SD=27) and 34 (SD=29) respectively. Patients' acceptance was optimal with missing items ranging from 0 to 1.2%. Out of 36 possible correlations among the checklist items: 81% were weak (r<0.40); 19% moderate (r=0.40-0.60) and none was strong. The checklist was able to discriminate between gender, showing significant worse scores for female in all symptoms except for diarrhea and skin problems (P<0.05). The checklist was also able to capture statistically significant different profiles (P<0.05) for patients with associated co-morbidity. *Conclusions.* The use of this 9 items checklist is feasible in CML patients and it targets important side effects of IM treatment. Further development is ongoing in a larger international setting within the EORTC Quality of Life Group to eventually devise an EORTC CML questionnaire.

0466

RISK FACTORS FOR INEFFECTIVE COPING IN ACUTE LEUKEMIA PATIENTS

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Background. Coping with cancer, in general, and acute leukemia, in particular represents a dynamic process influenced by medical, psychological and social issues. Many psychooncologists emphasize that some patients adapt better than others and psychosocial support services cannnot yet be offered to every patient. For this reason it is important to determine wich patient is at greatest risk of developing adjustment problems in order to offer them psychological support. *Aims.* Identification of risk factors for developing an ineffective coping in acute leukemia patients *Methods.* We have interviewed 100 acute leukemia patients in various disease stages and various therapeutically steps treated in the Hematology Department Clinic, Cluj-Napoca during November 2007-May 2009. Patient interviews were conducted using a questionnaire divided in an introductory part (consisting in obtaining informed consent and explaining the reason of the intervew) and 10 specific sections. The following topics were pursued: analysis of the way the diagnosis was communicated and the patient`s psychological reaction to diagnosis; the five-stage process of coping with the malignant diagnosis (denial, anger, bargaining, depression, acceptance); identification of factors with a facilitating effect in overcoming an ineffective coping and developing an effective coping; interval from diagnosis to acceptance; the level of acceptance; evaluation of patient internal and external resources; understanding different types of coping with malignant disease symptoms and therapy side effects; coping with substitution therapy with blood and blood products; coping with death of a fellow patient; taking self-blame for the disease; disease effect on patient's life on a personal, family, professional and social level; family behavior regarding the disease; self-assessment of coping. Based on the interviews, we evaluate the efficiency of coping (from a psychosocial counselor's and a haematologist's perspective) and the statistical significance of correlations between coping and 25 other variables (such as patient personality traits, patient`s demographic characteristics, disease-related and treatment-related issues, diagnosis communication, diagnosis acceptance, time passed to diagnosis acceptance, isolation in hospital, patient's psychological resources, selfblaming for the disease and others). Data was analyzed using SPSS. Statistical significance for various qualitative variables association was evaluated using the Pearson's chi-square test and Fisher Freeman-Halton's exact test in cases when at least 20% of the expected count less than 5. The study has the agreement of Ethic Committee of the Medicine and Pharmacy University Cluj-Napoca, Romania. *Results.* Risk factors for inefficient coping in acute leukemia patients are: lack of sense of humor (OR=12.6, 95% CI: 1.45-109.39); lack of fighting spirit (OR=23.33, 95% CI: 3.94-138.098). Inefficient coping in acute leukemia patients is statistically significant associated with; lack of sense of humor (P=0.008<0.05, statistically significant); lack of fighting spirit (P=0.0005<0.05, statistically significant); hopelessness/ helplessness (P=0.00031<0.05, statistically significant); low acceptance of diagnosis, i.e. values between 4 and 6 on a 1 to 10 scale (F=7.13, P=0.019<0.05, statistically significant) *Conclusions.* Analising 25 factors, only lack of humor and lack of fighting spirit representing risk factors for inefficient coping, so that these patients should benefit of targeted and individualised psychological support.

0467

STUDY OF EFFECTIVENESS RECOMBINANT HUMAN ERYTHROPOIETIN IN LYMPHOPROLIFERATIVE DISORDERS PATIENTS WITH ANEMIA

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Background. Anemia in patients with lymphoproliferative disorders is a frequent symptom and can influence the efficacy of antitumor chemotherapy, survival rate and overall quality of life (QoL). Red blood cell (RBC) transfusions are routinely used to treat anemia, while recombinant human erythropoietin (rHuEPO) treatment has been shown to significantly increase hemoglobin (Hb), reduce the number of RBC transfusions and improve QoL in patients with chemotherapy induced anemia. *Aims.* This study was find out the effectiveness Recombinant Human Erythropoietin in lymphoproliferative disorders patients with anemia and improving QoL. *Methods.* There were done this interventional prospective study to investigate the effectiveness of rHuEPO (epoetin alpha) on Hb concentration, RBC count and QoL in patients (n=27) with low-grade non-Hodgkin's lymphoma (n=7), chronic lymphocytic leukemia (n=8) and multiple myeloma (n=12). The median age of patients was 65.5 years (range 24-80). Recombinant human erythropoietin alpha was injected subcutaneously on 10.000IU 3 times a week. Before start of rHuEPO treatment all patients have being received two or more cycles of chemotherapy. The target Hb level was 12 g/dL and planned duration of treatment with rHuEPO within 16 weeks. QoL was assessed using the FACT-Anemia (FACT-An) questionnaire. *Results.* Mean baseline Hb concentration was 8.21±1.96 g/dL and RBC count was $2.63\pm0.68\times10^{12}$/L. The period of rHuEPO-therapy was from 6 to 16 weeks (mean 10±4 weeks and median follow-up of 9 weeks). During the study period, the Hb concentration and RBC count increased from baseline to 10.67±2.82 g/dL (P<0.02) and $3.38\pm0.98\times10^{12}$/L (P<0.01), respectively. An Hb increase >1 g/dL was observed in 17 patients (63%), while a non-response was observed in 37%. 4 patients (15%) needed the dose increase (from 10.000IU to 20.000IU), but only in 1 patient (4%) was positive response (Hb concentration increased on 3,4 g/dL during 16weeks). FACT-An demonstrated that rHuEPO-therapy reduced symptoms such as: fatigue, force and physical efficiency, depression, drowsiness, giddiness, headaches, pain in thorax and dyspnea. On a scale from 0 to 4 points, the symptoms reduced from 1.70±0.88 to 1.27±0.83 indicating an improvement in QoL in the study patients (P<0.05). We disclosed arterial hypertension (increasing 20 mm/Hg or more) in 6 patients with positive response (22.2%) and 1 case in non-response patients (3.7%) on rHuEPO-treatment. But this adverse event was controlled by administration of antihypertensive therapy and in 1 case by withdrawal of rHuEPO-treatment. Hb levels and arterial pressure were monitored weekly. *Conclusions.* The study has shown that rHuEPO is effective at increasing Hb and improving QoL in a small group of anemic patients with lymphoproliferative disorders.

0468

COMPARISON OF SYMPTOM BURDEN (PAIN, ANXIETY, DEPRESSION) IN PATIENTS WITH LEUKEMIA VERSUS PATIENTS WITH SOLID TUMORS AT DIAGNOSIS: IDEAL CUT-OFF POINTS FOR SCREENING BY ESAS

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Background. Contrary to what is generally accepted, not only solid cancer but also leukaemia patients may suffer from physical pain and emotional distress since diagnosis and during all phases of the disease (Morselli et al., Leu Res 2010 34:e67-8). *Aim*. We prospectively reviewed data by Hospital Anxiety and Depression Scale (HADS), Edmonton Symptoms Assessment System (ESAS), and Numerical Rating Scale (NRS) of ESAS, to compare the frequency and burden of physical pain, emotional distress and other symptoms, in patients with leukaemia, since diagnosis, during the neutropenia phase (+15), and at discharge,

during the various clinical phases (induction, subsequent consolidation, and autologous and allogeneic bone marrow-BM-/peripheral blood stem cell-PBSC- transplantation procedure), as well as in patients with non haematologic cancers, evaluated at diagnosis, as controls. *Patients and Methods.* 81 patients with acute leukaemia (66 myeloid and 15 lymphoblastic; 55 male, 26 female; median age 59 years-range 21-79-) and 118 patients with solid cancer; 51 female, 67 male; median age 65 years-range 42-84-) were enrolled. The diagnosis of depression and/or anxiety and moderate/severe depression and/or anxiety were made when patients scored 8 or more, and 11 or more in HADS questionnaire, respectively. The sensitivity, specificity, positive and negative predictive values for ESAS were calculated. *Results.* According to the HADS score, during the induction phase, depression was reported in 25.7%, 37.3% and 26.9% of the patients, at diagnosis (time 0), at +15 and at discharge, respectively, while anxiety in 32.4%, 36.9%, 30.8% of the cases, at the same time intervals, respectively. Depression was reported in 31.1% while anxiety in 35.6% of all questionnaires, respectively, collected from patients at all time intervals during all clinical phases. According to the NRS score, during the induction phase, mild pain was reported in 36%, 30%, and 15% of the cases, at diagnosis, at +15 and at discharge, respectively, being moderate to severe pain reported in 14%, 18% and 6% of the cases, at the same three time intervals, respectively. A cut off of 2 out of 10 or more in the ESAS gave a sensitivity of 71% and 95% with a specificity of 76% and 69% for depression and moderate/severe depression, respectively, when questionnaires during the induction phase were considered, while a sensitivity of 60% and 78% with a specificity of 70% and 67%, when questionnaires at all time intervals, during all clinical phases were considered. A cut off of 3 or more in the ESAS gave a sensitivity of 73% and 86% with a specificity of 84% and 75% for anxiety and moderate/severe anxiety, respectively when questionnaires during the induction phase were considered, while a sensitivity of 51% and 74% with a specificity of 84% and 78% for anxiety and moderate/severe anxiety, respectively, when questionnaires at all time intervals, during all clinical phases were considered. Symptom severity appeared similar (pain, anxiety, depression and dyspnea) or even higher (fatigue, nausea, drowsiness, appetite, well-being, sleep) in leukaemia compared with solid cancer patients at diagnosis. *Conclusions*.We propose the first ideal cut-off points of ESAS for screening of depression and anxiety in leukaemia patients at diagnosis and during all clinical phases, out of the palliative care.

0469

SINGLE DOSE PALONOSETRON IN PREVENTING CHEMOTHERAPY-INDUCED NAUSEA AND VOMITING IN PATIENTS WITH HODGKIN LYMPHOMA RECEIVING ABVD REGIMEN

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Background. Chemotherapy-induced nausea and vomiting (CINV) is associated with a significant deterioration in quality of life. The ABVD regimen (Adriamycin, Bleomycin, Vinblastine and Dacarbazine) is considered the standard of care for first line Hodgkin Lymphoma and patients treated with this regimen have a high risk of CINV. *Aims.* The aim of our study was to evaluate the efficacy of a new agent, palonosetron, in Hodgkin's lymphoma patients treated with ABVD regimen. Palonosetron is a second-generation 5-HT3 receptor antagonist (5HT3RA) with a longer half-life and a higher binding affinity than old 5-HT3RA. *Methods.* All patients were treated with palonosetron 0.25 mg i.v. and dexamethasone 8 mg i.v. prior chemotherapy administration. Complete Response (CR: no vomiting and no rescue therapy), during the overall phase (0-120 hours) of the first ABVD cycle, was the primary endpoint. Emesis-free and use of rescue medication rates, during the acute (0-24 hrs) and overall phases, were the secondary endpoints. Incidence of nausea according Likert scale was evaluated during the study observation period. Two ABVD cycles were evaluated. *Results.* From January 2008 to February 2009 36 patients were enrolled, most of the patients were male (63.9%) and young (median age 33.5). All patients received study treatment during the first cycle of ABVD, while 9 of them changed antiemetic prophylaxis at the second cycle. The primary endpoint (CR 0-120 hours) was achieved by 55.6% of patients. Most of the patients didn't experience emesis during the overall phase of the first (86.1%) and the second (85.2%) ABVD cycle. 61.1% and 88.9% of patients didn't use rescue medication during the first and the second ABVD cycles respectively. Administration of palonosetron was well tolerated and the most common adverse events were transient constipation and headache as expected. *Conclusions.* In conclusion our study demonstrated that a single dose palonosetron (0.25 mg) and a single dose dexamethasone (8 mg) on day 1 was effective in preventing CINV in Hodgkin Lymphoma patients treated with ABVD regimen.

0470

USE OF CHEMOTHERAPY, IMMUNOTHERAPY AND SUPPORTIVE CARE IN THE END OF LIFE OF PATIENTS TREATED FOR B-CELL DIFFUSE LARGE CELL LYMPHOMA IN A SINGLE INSTITUTION: RETROSPECTIVE ANALYSIS OF A COHORT OF 410 PATIENTS AND TRENDS ACCORDING TO THE TIME PERIOD

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Background. Despite major improvement in the outcome of diffuse large B cell lymphoma (DLBCL), some patients will die from direct evolution of the disease. Adequate timing for cessation of aggressive care is difficult to establish. *Aims.* To document in a cohort of patients treated in a single institution for DLBCL, the management during the last month of life. *Patients and methods*. From 1996 to 2008, 410 pts older than18 years have been referred in our institution for first line therapy of DLBCL. Patients with primary cerebral lymphoma and seropositive for HIV were excluded.. Median age was 64 years. With a median follow-up of 60 months, 5-year overall survival and 5-year EFS were respectively 67% and 61%.132 pts died including 81 pts from terminal evolution of the lymphoma, 27 pts from toxic deaths in first line, 9 pts from other solid tumours (8) or myelodysplasic syndromes (1), and 15 pts from other or unknown causes. We have analyzed these 81 pts died from direct evolution of the disease and their management in the endlife according 2 time periods for initial diagnosis 1996-2002 (cohort 1) and 2003-2008 (2003-2008). *Results.* Median age at diagnosis was 69.5 years (24-89) and median age at death was 70 years (25-90). 53% of pts were stage IV and 50% had IPI>= 3. Median time between diagnosis and death was 13 months (1.5-106). Forty% of pts died in our institution, 25% died in a homecare setting, 6% in an intensive care unit and 29% in other institutions mainly in palliative care units with a similar repartition of death place according to the time period. Except for the use of Rituximab, there were no significant differences in the endlife management of these patients. A pharmacoeconomic evaluation will be reported. *Conclusion.* This is the first study reporting the incidence of 'aggressive care"the last month of life of patients with evolutive DLCBL.Prognostic factors are needed in order to better predict risks and benefits of subsequent therapy in order to avoid useless and expensive treatments and to optimize resources for palliative care.

Table.

		Total	cohort 1	cohort 2
		81	42	39
Number of chemotherapy regimen:				
	0-2	44	21	23
	3	23	13	10
	> 3	14	8	6
Median time from last chemotherapy to death (day)		38	35	48
% of pts receiving Rituximab at least one time		60%	33%	92%
% of pts receiving chemotherapy last month		40%	33%	45%
% of pts receiving Rituximab last month		14%	2%	26%
%of pts receiving platelets last month		20%	21%	18%
% of pts receiving erythocytes last month		26%	24%	28%
% of pts receiving morphine last month		59%	55%	67%
% of pts receiving antibiotics last month		27%	33%	21%

0471

THE VALUE OF PATIENT ASSOCIATIONS IN HEMATOLOGY CLINICAL RESEARCH: CHALLENGES AND PRELIMINARY RESULTS OF A JOINT STUDY INVESTIGATING HEALTH OUTCOMES IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA.

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Background. Involving patient associations and advocacy groups in cancer research and healthcare planning is now regarded as key issue, but there is lack of evidence in hematology research. *Aims.* To describe methods, challenges and preliminary results of a joint quality of life

(QoL)-based study between a research organization and a national patient association (i.e. the Italian Association against Leukemias, Lymphoma and Myeloma-AIL). *Methods.* Patients with CML in treatment with Imatinib (IM) for at least three years were approached and invited by their treating physicians (i.e. investigators) in the Hospital. All consenting patients were then handed over a QoL Survey Packet with the request to completing it at home and send it back with a pre-paid reply envelope. AIL voluntaries involvement was expected to maximize patients compliance in returning the Survey and provide logistic support to the conduct of study. Participating local AIL-based branches were located in the same cities of research centers, whereas possible, and a responsible AIL representative, in each branch, was appointed to collaborate with local investigators. Prior to study initiation, an educational session describing research protocol, study purposes and logistics was organized providing all actors involved (investigators and AIL representatives) standardized working guidelines. At study closure, an ad hoc survey was completed by all AIL representatives. The survey covered a number of areas including: socio-demographics, challenges encountered in terms of relationships with investigators and related logistical barriers and level of satisfaction for being involved in the study. *Results.* Twenty-six research centers throughout Italy participated in the study and 473 patients were enrolled between March to December 2009. Patients' compliance in returning valid QoL Survey Packet was 94% which is particularly high compared to similar research settings in terms of study design and patient population (i.e. mid to long term survivors). Twenty-three AIL representatives were involved: 3 male (13%) and 20 female (87%). 43% had high school diploma and 57% had a university degree and nearly all (95%) have never participated in previous collaborative studies. As concern the topics addressed during the "educational session", organized prior to study start, these were rated as "very relevant"for study purposes by 68% of participants; all of them (100%) found working guidelines as "very helpful". Frequency distribution to the question: "to what extent this research experience was gratifying to you?"was as follow: not at all (0), a little bit (4%), somewhat (41%), quite a bit (45%) and very much (9%). All of them would participate again in similar studies and 75% would be happy to play even a more active role in future studies dealing with QoL issues. Nearly all (95%) believed the optimal patients' compliance rate achieved was, to some extent, attributable to their collaboration in this research. Only 35% judged their contribution in reducing investigators' workload being minimal. *Conclusions.* Patient associations can potentially be of great value in QoL-based studies in hematology, providing tasks are clearly defined a priori. Future experiences are encouraged to provide additional data in this area.

0472

INVESTIGATION OF PSYCHIATRIC FACTORS AFFECTING COMPLIANCE OF TRANSFUSION DEPENDENT THALASSAEMIA PATIENTS WITH IRON CHELATING AGENTS, DESFEROXAMINE, DEFERIPRONE AND DEFERASIROX

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Introduction: Transfusion-dependent thalassaemia patients' compliance with iron chelation therapy is the determining factor for complication-free survival and better quality of life. It is known that depression seriously influences compliance with therapy; however, depression potentially covers other psychiatric factors negatively affecting compliance, such as affective disorders, behavioural disorders, paranoid ideation or psychotic symptomatology. *Objective.* To investigate the potential existence of psychiatric factors which could hide under depression and contribute to poor compliance, in patients following different iron chelation regimens: Desferoxamine (DFO), Deferiprone (DFP) or Deferasirox (DFX) monotherapy, and combination therapy with DFO and DFP. *Patients and Methods.* 47 patients, 21 males and 26 females, mean age 38 years (range 27-56) from one thalassaemia unit were enrolled in the study. Patients fell into four groups according to iron chelation regimen: Group A, 14p receiving DFO (40 mg/kg x 24h x 6 days/week); Group B, 11p formerly DFO, switched to DFP (75 mg/kg/day); Group C, 11p on combined treatment with DFO (40 mg/kg x 24h x 4 days/week) and DFP (75 mg/kg/day); and Group D, 11p switched from DFO or DFP to DFX (30 mg/kg/day). Each patient was interviewed by the same doctor. Compliance was estimated by the compliance index. Psychiatric parameters were evaluated by the Brief Psychiatric Rating Scale (BPRS), a simple and reliable tool for recording certain psychiatric symptoms. Total BPRS score ≤40 indicates no serious psychopathology. Psychological support was provided by experienced staff of the clinic, while psychiatric treatment protocols did not change during the study. *Results.* Compliance differed significantly between groups (P=0.003) and was best in Group D (DFX). BPRS score > 40 was detected in 87% of all patients in the study. Psychotic behavior was observed in one patient, who did not comply with parenteral or oral chelation treatment. A high rate of depression (85%) was found. Anxiety disorders, panic attacks, paranoid ideation and inappropriate affect associated with medium and poor compliance were recorded in high percentages in Groups A, B, and C. However patients in Group D (DFX) who all had excellent compliance, showed rates of depression (77%), anxiety (91%), panic attacks (91%), hypomania (45%), paranoid ideation (45%) and inappropriate affect (55%) as high as or higher than other groups (Table 1). *Conclusions.* This study showed that thalassaemia patients had serious psychopathological characteristics underlying depression, potentially affecting their compliance with iron chelation therapy. However, these parameters did not negatively influence compliance with a specific regimen. Severe depression associated with poor compliance might mean masked suicide attempts. Further studies in a larger sample will be required to elucidate association between the occurrence of psychiatric factors and the treatment regimen.

Table 1. Psychopathology by compliance with iron chelation therapy.

	Group/Iron chelation treatment											
	A (n=14)/ DFO			B (n=11)/DFP			C (n=11)/ DFO + DFP			D (n=11)/DFX		
	E*	M	P	E	M	P	E	M	P	E	M	P
Compliance	4	7	3	8	1	2	6	4	1	11	0	0
Psychiatric Parameters												
Depression /hypochondriasis	2	6	3	4	1	2	4	3	1	8	0	0
Anxiety disorder	0	5	3	0	1	2	0	4	1	10	0	0
Panic attack	0	5	3	0	1	2	0	2	1	10	0	0
Hypomania	1	2	0	1	1	2	1	3	1	5	0	0
Paranoid ideation	0	4	3	0	1	2	0	4	1	5	0	0
Inappropriate affect	0	4	3	0	1	2	0	4	1	6	0	0

* E: excellent, M: moderate, P: poor

0473

NEW INSTRUMENT FOR COMPREHENSIVE SYMPTOM PROFILE ASSESSMENT IN PATIENTS WITH MALIGNANT LYMPHOMAS: APPLICABILITY AND CHARACTERISTICS

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Comprehensive symptom assessment before and during treatment in lymphoma patients is worthwhile. We aimed to develop a new symptom assessment tool - Comprehensive Symptom Profile in Lymphoma Patients (CSP-Lym) and test its applicability in this patient population. CSP-Lym is being developed to assess the severity of 45 symptoms specific for lymphoma patients. It consists of numerical rating scales, scored from "0"(no symptom) to "10"(most expressed symptom). Thirteen clusters of symptoms have been identified, which were clinically relevant and increased the practicability of the tool. Applicability of CSP-Lym with preliminary analysis of psychometric properties was tested in a pilot study. Thirty one patients with different types of malignant lymphomas (Stage - II-IV) were included in the study: Non-Hodgkin's lymphoma - 8; Hodgkin's lymphoma - 23. Mean age was 34 years old; male/female distribution -14/17. The utility of CSP-Lym was demonstrated: all the items were easy for the patients to read and understand; the data produced by the tool were clear for interpretation by physicians and were used by them in clinical decision making. Reliability of CSP-Lym was satisfactory (Chronbach's alpha coefficient varied from 0.60 to 0.98). The construct validity of CSP-Lym was proved by factor analysis and "known-group"comparison. Statistically significant differences (P<0.05) in symptom severity were found in the groups with different patient status. Sensitivity to changes was demonstrated by comparison of symptom severity before and after treatment. Thus,

CSP-Lym is an appropriate and practical tool to assess the symptom severity in lymphoma patients. The utility of the questionnaire was shown; preliminary psychometric properties appeared to be satisfactory. Further studies are needed before the wide-spread use of CSP-Lym in clinical practice and clinical trials.

0474

GERMAN G-DRG LUMP SUM SYSTEM IS CAPABLE TO RECOVER THE TREATMENT COSTS OF PATIENTS (PTS) WITH ACUTE MYELOID LEUKEMIA (AML) RECEIVING S-HAM INDUCTION CHEMOTHERAPY (CTX) - BUT NOT FOR NON-RESPONDERS

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*Background.*Sequential CTX with high dose cytarabin and mitoxantrone (S-HAM) as induction treatment under evaluation in phase III trials and might become one new standard of care for patients with de-novo AML. The regimen was shown to shorten the duration of critical neutropenia by 14 days as compared to standard double induction, which might have an impact on hospital costs as well. In Germany inpatient treatment is remunerated by DRG lump sums based on national average costs for AML patients receiving double-induction chemotherapy (G-DRG R60A). However hospitals report that the individual treatment costs show a high variance and little is known about the variables responsible for cost differences or whether the G-DRG lump sum compensations are cost covering. *Aims.*By comparing the costs and the revenues for AML treatment of all pts treated with S-HAM in a five year period we wanted to identify predictive clinical parameters for high expenditures and evaluate the ability of G-DRG lump sum system to bear the costs of a very complex treatment such as induction CTX. *Methods.*This is a single institution cost-benefit-analysis in a large referral university hospital consecutively analyzing 77 pts with AML receiving S-HAM from 2004-2008. Revenues were retrieved from hospital bills. Costs where valued from hospital perspective and transferred in a two dimensional 110-field cost-unit accounting matrix for each patient. Univariate and multivariate analyses were run to identify diagnoses and clinical procedures to be discriminators for cost-benefit-differences. *Results.*Median length of hospitalization was 47 days and mean *per diem* costs were 0,864€. Mean revenues were 44,6T€ (range 16,3T€;148,2T€) and mean costs were 45,7T€ (range 10T,5€; 173,4T€). DRG remuneration was almost cost covering for the cohort (total loss -83,9 T€) but not less then 32 pts showed a negative cost-benefit-ratio (mean loss -15,0T€). 41 pts showed a positive cost-benefit-ratio (mean win 9,7 T€). Cost-Benefit-ratio was not different with respect to age (P=0.31), survival (P=0.08), mechanical ventilation (P=0.26) or the use of pretended high cost drugs like antifungal agents (P=0.70). However non-responders (P=0.02) and patients requiring intensive care in general (P=0.03) where significantly at higher risk for insufficient financial recovery. *Summary/Conclusions.*G-DRG lump sum system is capable to recover the treatment costs of AML S-HAM CTX but a high individual financial risk remains. Non-responders are the patient group with the highest difference between allocated costs and retrieved revenues.

0475

KOBRA - A NEW AUTOMATICALLY GENERATED SCORING SYSTEM IS PREDICTIVE FOR TREATMENT COSTS OF PATIENTS (PTS) WITH ACUTE MYELOID LEUKAEMIA (AML) RECEIVING S-HAM INDUCTION CHEMOTHERAPY (CTX)

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*Background.*Sequential CTX with high dose cytarabin and mitoxantrone (S-HAM) was shown to be highly effective as induction treatment in a phase II study and is therefore currently being evaluated in a phase III trial for patients with *de novo* AML. Many studies in health economics use *per diem* rates to estimate the costs of inpatient treatment. Cost estimates are often based on experts' opinions and rest rarely on real life data. It remains unclear if the *per diem* rates can be used in a complex setting like induction CTX of AML patients or if more sophisticated scores are mandatory. If more complex scores would be necessary for cost prediction an automated generation of these scores would be desirable. *Aims.*We calculated real life costs for AML patients and compared *per diem* rates with scoring systems based on TISS and SAPS. We established a predictive score system based on electronically available laboratory data. We presumed that laboratory values that are characteristic for organ dysfunctions might be the best discriminators for costs. *Methods.*The study was a single institution retrospective chart review in a large referral university hospital analyzing 60 pts with AML receiving S-HAM from 2004-2008. Costs where valued from hospital perspective and transferred in a two dimensional 110-field cost-unit accounting matrix for each patient. TISS-28, TISS-10 and SAPS scores where assessed by hospital chart abstraction for each day of treatment. KOBRA score was established by daily ranking of laboratory values 4 predefined classes scored with 0;1,2;4 points per day for CRP, leucocytes, thrombocytes, creatinine and bilirubin for each patient and day. When missing, the newest available value was used. *Results.*The use of *per diem* rates (860 €/ day) showed an apparent correlation (r^2=0.53) but a better correlation could be achieved with daily SAPS-2 and daily TISS28 score (r^2=0.77). KOBRA score (142€/ point) was reliably predictive for treatment costs (r^2=0.67). *Summary/Conclusions.*Treatment costs strongly correlate with diagnostic and therapeutic sores, with TISS-28 supposing to be one of the most predictive. The KOBRA score can be generated automatically and thus can be used as a pragmatic and effective predictor for treatment costs in clinical and economical trials.

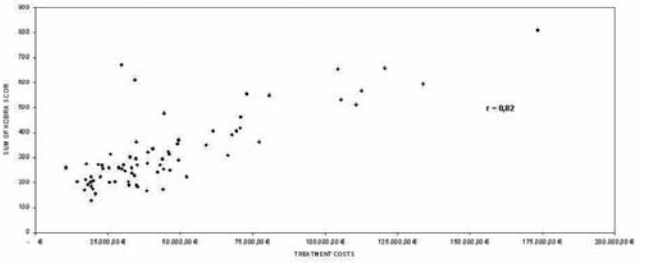

Figure. Correlation between KOBRA and TREATMENT COSTS

0476

COMPLETE RESPONSE AND SURVIVAL OUTCOMES WITH BORTEZOMIB-MELPHALAN-PREDNISONE: AN INDIRECT COMPARISON VERSUS THALIDOMIDE-BASED REGIMEN FOR THE FRONT LINE TREATMENT OF MULTIPLE MYELOMA

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Background. Melphalan and Prednisone (MP) has been considered a standard of care for front line treatment for multiple myeloma (MM) patients that are not eligible for high-dose chemotherapy and autologous stem cell transplant. Both Bortezomib-Melphalan-Prednisone (VMP) and Melphalan-Prednisone-Thalidomide (MPT) combinations have been approved in Europe in this patient group. Thalidomide with Cyclophosphamide and Dexamethasone attenuated (CTDa) is a regimen commonly used in clinical practice in the UK. There has been no head-to-head trial directly comparing VMP to MPT and CTDa. *Aim.* To compare the efficacy of VMP with MPT and CTDa as front line treatment of MM in patients non eligible for transplant via an indirect comparison. *Methods.* Seven randomized controlled trials investigating the efficacy of MPT (3 published articles, 2 abstracts comprising 1495 patients), VMP (1 published article, 682 patients) and CTDa (abstract, 523 patients) relative to MP were identified through a systematic literature review up to May 2009. Endpoints of interest were complete response (CR), overall survival (OS) and progression free survival (PFS). The relative efficacy estimates of MPT versus MP as obtained from the MPT-MP trials were combined using meta-analytic techniques and simultaneously indirectly compared with the relative efficacy of VMP versus MP and CTDa versus MP. Published Kaplan Meier curves were digitally scanned to reconstruct life tables and facilitate the analyses for OS up to 48 months and PFS up to 30 months. This adjusted indirect comparison was performed with Bayesian fixed (CR) and random effects models (OS, PFS). As compared to frequentist approach, Bayesian meta-analysis offers a more informative summary of the likely value of efficacy after observing the data and allows for direct probabilistic inferences. *Results.* Our indirect analysis demonstrated that patients treated with VMP were more than two times as likely to achieve a CR compared to patients treated with MPT (Relative Risk=2.64; 95% CrI: 1.32-5.10). The relative risk for VMP versus CTDa was 1.57 (95% CrI: 0.74-3.33). Both VMP and MPT were superior to MP

for PFS (HR=0.49, 95% CrI 0.35-0.68 and HR=0.65, 95%CrI 0.55-0.78 respectively) and OS (HR=0.68, 95% CrI 0.51-0.89 and HR=0.83, 95%CrI 0.71-0.97, respectively) (Table 1); the indirect comparison of VMP versus MPT resulted in a hazard ratio (HR) of 0.75 (95%CrI 0.52-1.09) for PFS and a HR of 0.81 (95%CrI 0.59-1.12) for OS, indicating a trend towards improved survival outcomes with the VMP combination. The HR for CTDa versus MP was 0.92 (95%CrI 0.67-1.28) for PFS; the indirect comparison results in a HR of 0.54 (95% CrI 0.33-0.84) for VMP versus CTDa. The probability of superiority of VMP over MPT was 93% for PFS and 72% for OS. *Conclusions.* This indirect comparison of VMP versus MPT as front line treatment in myeloma patients not eligible for transplant has shown that VMP results in a twofold higher CR rate than MPT. This translates into a trend towards improved progression and survival outcomes with VMP versus MPT. The indirect comparison of VMP versus CTDa results in a superior PFS and a trend towards improved OS with VMP.

Table 1. Hazard ratios for overall suvival.

Randomised clinical trials	Overall Survival as reported in individual trial HR (95% CI)	Overall Survival as estimated with piece-wise constant hazard model HR (95% CrI)	
		Individual trial	*Meta-analysis*
		VMP vs. MP	
San Miguel 2009	0.65 (0.51-0.84)	0.68 (0.53-0.86)	**0.68 (0.51-0.89)**
		MPT vs. MP	
Facon 2007	0.56 (NR)	0.57 (0.41-0.78)	
Hulin 2009	0.68 (NR)	0.67 (0.46-0.96)	
Palumbo 2008	1.04 (0.76-1.44)	1.09 (0.79-1.51)	**0.83 (0.71-0.97)**
Wijermans 2009	NR	0.82 (0.62-1.09)	
Gulbrandsen 2008	NR	1.14 (0.86-1.52)	
		CTDa vs. MP	
Morgan 2009	0.98 (0.80-1.21)	0.94 (0.71-1.25)	0.92 (0.66-1.28)
		VMP vs. MPT	**VMP vs. CTDa**
Indirect comparison		**0.81 (0.59-1.12)**	**0.74 (0.47-1.11)**

0477

ECONOMIC EVALUATION OF CASPOFUNGIN VERSUS LIPOSOMAL AMPHOTERICIN B FOR EMPIRICAL ANTIFUNGAL THERAPY IN PATIENTS WITH PERSISTENT FEVER AND NEUTROPENIA IN SWEDEN

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Background. Prolonged neutropenia in patients undergoing major cancer chemotherapy increases the risk of infection. Liposomal amphotericin B has frequently been used for empirical antifungal therapy in patients with neutropenia and persistent fever despite antimicrobial treatment. In a randomized, double-blind, multinational trial, caspofungin was found to be as effective as liposomal amphotericin B and generally better tolerated. Costs for the empirical antifungal therapy associated with caspofungin and liposomal amphotericin B, respectively, might differ and incremental cost-effectiveness ratios for caspofungin versus liposomal amphotericin B in Sweden remain to be explored. *Aims.* The aim of this study was to evaluate the cost-effectiveness of caspofungin versus liposomal amphotericin B for empirical antifungal therapy in patients with persistent fever and neutropenia in Sweden. *Methods.* A decision-analytic model was constructed to evaluate the cost-effectiveness of caspofungin versus liposomal amphotericin B. Model outcomes included life years lost (LYL), quality adjusted life years (QALYs) lost, expected antifungal drug costs, expected other medical costs (hospitalization costs and drug costs for adverse events), and expected overall costs. Incremental outcomes, defined as the differences between the two treatment arms, were then calculated for QALYs saved, life years saved and incremental costs to ultimately establish incremental cost-effectiveness ratios. Efficacy and tolerability data were based on additional analysis of a randomized, double-blind multinational trial. Information on life expectancy, medical resource use and costs were gathered from the literature and expert opinion. Probabilistic sensitivity analysis was used to incorporate uncertainty in the model. Results are reported as point estimates with uncertainty reflected by the 2.5th and 97.5th percentile of the uncertainty distribution. *Results.* The caspofungin total treatment costs amounted to €22,948 (95% uncertainty interval 22,125; 23,813) whereas the liposomal amphotericin B treatment cost amounted to €26,684 (25,815; 27,555), a difference of €3,736 (3,077; 4,318). Treatment with caspofungin resulted in 0.25 (0.01; 0.55) QALYs saved in comparison to treatment with liposomal amphotericin B. In Figure 1 the uncertainty of the QALYs and cost estimates for caspofungin relative to liposomal amphotericin B are presented. Taking into account the uncertainty in the results, there is a >95% probability that caspofungin is economically dominant, i.e. cost saving and QALY saving. *Conclusions.* Given the underlying assumptions and data used, our evaluation demonstrates that caspofungin is cost-effective compared to liposomal amphotericin B for empirical antifungal therapy in patients with persistent fever and neutropenia in Sweden.

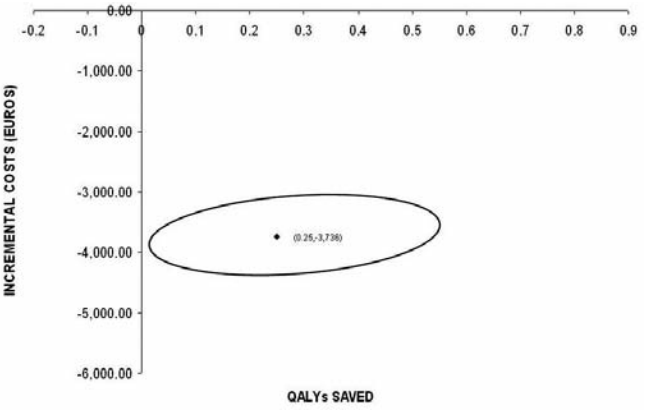

Figure 1. Cost effectiveness plane (incremental costs vs. quality adjusted life years (QAl, Ys) saved with caspofungin relative to liposomal amphotericin B).

0478

NEED OF RECONSIDERATION OF MOBILIZATION STRATEGY IN AUTOLOGOUS STEM CELL TRANSPLANTATION FOR MULTIPLE MYELOMA: RESULTS OF THE POSITIVE IMPACT OF HIGH NUMBER OF CD34+ INFUSED CELLS

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Background. Autologous Stem Cell Transplantation (ASCT) with Peripheral Blood Stem Cells (PBSC) is widely used in first line treatment for Multiple Myeloma (MM) patients. The rapidity and stability of cell engraftment may correlate with the number of CD34+ cells in the autograft. However, the impact of this number on hematologic recovery and long term outcomes is not very well defined yet. Having a higher number of collected CD34+ cells seems to be a very important and accessible objective in 2010 (availability of mobilization agents), also it has in the same time cost and efficacy results. *Material and Methods.* This study concerned 130 MM patients who underwent ASCT in our center between years 2000 and 2007. There were 79 M and 51 F with a median age of 56.8 years (34-72). At diagnosis there were 71 IgG (49κ, 22λ), 26 IgA (15κ,11λ), 2 IgD (1κ, 1λ), 27 light chain (18κ,9λ), 2 plasma cell leukemia and 2 nonsecretory. There were 11 patients in stage I (10A and 1B), 12 IIA, 96 III(75A and 21B) and 11 not classified. At diagnosis, 24 patients had del(13), and 65 had high levels of β2microglobulin. The median interval between diagnosis and ASCT was 7.8 months (3.5-131). Before transplantation, all patients received G-CSF 5µg/kg/day, PBSC were mobilized in steady state in 135 cases, 62 after G-CSF + cyclo. As conditioning, all pts received Mel. alone. Sixty six patients received a single ASCT and 64 patients received 2 ASCT in a double ASCT program. After transplantation, there were 2 graft failure, 40% of patients received red blood cell transfusions, and 64% received platelet transfusions. The median number of days with neutrophils <0.5G/L was 6 (0-33) and with platelets <20G/L was 17 (2-104). The median length of hospitalization for auto transplantation was 18 days (14-54). *Aim.* To assess the impact of the infused CD34+ cells number, we have analyzed 2 groups: group 1 (n=86) for ASCT with a number of CD34+ ≤3×10^6/kg and group 2 (n=107) for ASCT with a number of

$CD34^+$ >3×10^6/kg. For cost analysis, data were collected regarding the mobilisation and harvest of PBSC, and the graft period until hospital discharge. *Results.* We found a high significant impact of the high number of infused $CD34^+$(group 2) on platelets recovery (P=0.002), a trend for the high number of infused $CD34^+$ cells (group2) on leukocyte recovery O.R= 0.748 [0.5-1.0] (P=0.0568) and a high significant impact of the same group on neutrophils recovery O.R= 0.670 [0.5-0.9] (P=0.009). The multivariate analysis using Cox model showed a significant impact only of poor prognostic factors on overall survival O.R.= 7.94 [1.0-59.2] (P=0.04) and also on progression free survival (PFS) O.R.= 2.55 [1.1-5.7] (P=0.024). *Conclusions.* High level of infused CD34+ appeared to be very optimal for hematological recovery after ATSC in MM, without any significant impact on O.S and PFS. Our study showed that a high number of $CD34^+$ cells number had an important economical impact including a total cost saving of 1500 euros (P=0.03).

0479

COST-EFFECTIVENESS OF TREATMENT WITH RITUXIMAB IN PREVIOUSLY UNTREATED PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA IN ROMANIA

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Background. First-line treatment with rituximab in combination with fludarabine and cyclophosphamide (R-FC) compared with FC alone in patients with untreated CLL has shown to increase PFS and reduce the risk of progression or death. *Aims.* To evaluate the long-term outcomes and cost-effectiveness of R-FC versus FC from the Romanian National Health Insurance House perspective. *Methods.* A three health states transition Markov model (progression-free survival, progression and death) was developed. Patient level data was obtained from the ML17102 study (CLL-8) evaluating progression-free survival (PFS) and overall survival (OS) in patients with previously untreated CLL. PFS was modeled using a Weibull function. An equal risk of death for R-FC and FC patients following disease progression was assumed; OS was modeled as a Markov process with a constant rate of death, obtained by modeling post-progression to death as a single population. All-cause mortality was obtained from Romanian life-tables. Time horizon considered is 15 years. Utility values were drawn from published sources. Direct costs considered were drug acquisition, drug administration, adverse events and supportive care costs. Unit cost values were used from published sources in Romania. Costs were discounted by 5%, outcomes by 0%. Sensitivity analyses were performed. *Results.* This analysis showed that for each treatment where R-FC is used instead of FC, the additional cost per patient would be on average 15,313 Euro. Benefit to the patient would be 1.008 QALY, on average, for a 15 years time horizon. The additional cost per QALY gained for R-FC over FC would be 15,191 Euro. *Conclusion.* This study confirms the long-term clinical and economic benefits of adding rituximab to FC in patients with CLL in Romania.

0480

NEONATAL SCREENING OF NEWBORNS: TARGETED VERSUS UNIVERSAL APPROACH AS A COST EFFECTIVE STRATEGY

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Background. Hemoglobinopathies are quite common in the ethnically diverse Omani subjects and represent a major public health concern. Disease-oriented specific prevention and control programs are mandatory and particularly relevant in the context of the high consanguinity rates in this population. High performance liquid chromatography [HPLC] is a powerful tool to screen newborns for haemoglobinopathies. Neonatal screening includes cord blood samples collection, screening and follow up of all newborns with abnormal results. *Aims.* The study aimed at establishing neonatal cord blood screening in the Sultanate of Oman to determine the prevalence of haemoglobinopathies and recommend a cost-effective strategy to the health authorities. *Methods.* A total of 7837 consecutive cord blood samples were screened for the presence of the underlying haemoglobinopathies by HPLC using Biorad Variant II program between April 2005 & March 2007. Complete blood counts [CBC] were also obtained on Cell Dyn 4000 automated blood cell counter. All samples were then processed to isolate and store mononuclear leukocytes for subsequent confirmatory molecular diagnostics. *Results.* The findings indicated a 48.5% incidence of α-thalassaemia, based on significant amounts of Hb Barts on HPLC and low mean cell volume [MCV] & mean cell haemoglobin [MCH] on the CBC. Amongst the α-globin-related abnormalities, the overall incidence of other haemoglobinopathies was 9.5% (4.8% sickle cell trait, 2.6% β-thal trait, 0.9% Hb E trait, 0.8% Hb D trait, 0.08% Hb C trait, 0.3% sickle cell disease and 0.08% homozygous β-thalassemia). *Summary/Conclusions.* The significantly high incidence of haemoglobinopathies amongst the newborns in the Sultanate of Oman emphasizes the value of neonatal cord blood screening to be implemented as the first step in the national strategy towards the management of haemoglobinopathies including early diagnosis, comprehensive clinical care and counseling of the affected families. The results of this large study indicate that using HPLC & CBC[<2 USD/sample] and prescreening of parents to select only abnormal samples for neonatal cord blood screening <10% samples] can be recommended as a highly cost-effective method targeted to screen only the abnormal samples (Figure).

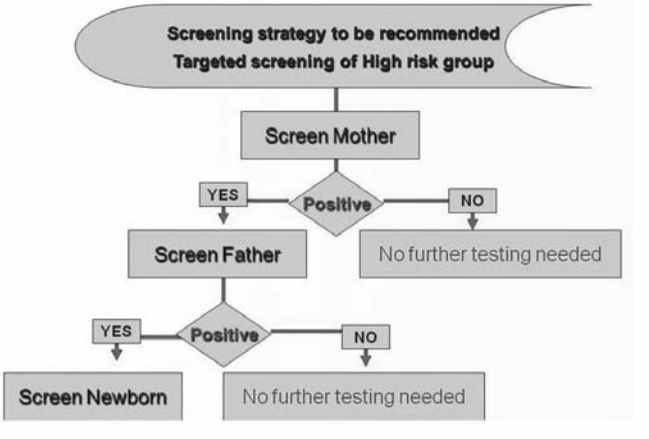

Figure. Approach to Targeted Newborn screening

0481

DO WE NEED EDUCATION PROGRAMS ABOUT IRON DEFICIENCY ANEMIA (IDA)?

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Background. Iron deficiency anemia (IDA) is the most common form of anemia, but usually it is not an end diagnosis, and requires complete work-up until the clarification of underlying cause . We felt that in our center to many missdiagnosed or not completely diagnosed patients have been sent to Hematologist without proper evaluation for IDA. So we tried to explore this impression and improve management of these patients. *Aims.* We evaluated influence of continuing medical education of general practitioners on managing the patients with IDA. *Methods.* During three years long, prospective study, altogether 1586 patients (983 in initial and 603 in final group) were referred to Hematology Outpatient Clinic, Rijeka University Hospital Center, Croatia due to diagnosis of IDA.They were examined by clinical hematologist during the first visit and follow up period. Patients were questioned by the means of questionnaire and complete laboratory analyses were performed in order to: evaluate physical condition and laboratory findings, to assess duration of anemia, possible other specialists' consultation, iron supplementation therapy, and finally, determine the type of anemia present. Initial group of 983 patients was examined during one year period. Following the education campaign the same parameters were analyzed in comparable (final) group of 603 patients during next one year period. *Results.* Following the education, the number of patients referred to Outpatient Clinic due to diagnosis of IDA was significantly decreased from 983 (61.97%) to 603 (38.02%) (P<0.05) as was the number of patients referred as having IDA but finally established to have a different type of anemia, from 661 (97.24%) to 149 (24.71%)(P<0.001). The number of patients started on iron supplementation therapy before establishing the type of anemia was significantly decreased from 543 (55.24%) to 76 (12.60%) (P<0.001) as well as duration of iron supplementation therapy administered in these cases (21±9.8 vs 6±8.7 weeks) (P<0.001). We have detected a significant decrease in: time necessary for definitive diagnosis (49±19.2 vs 28±9.1 weeks) (P<0.001), number of visits to other specialists (2.9±1.35 vs 1.1±0.94) (P<0.05), duration of anemia before treatment initialization (41±29.8 vs 26±18.7 weeks) (P<0.001). Average hemoglobin (Hg) level in patients referred to hematologist was significantly lower following education (98.9±15.5 vs 82.6±14.2) (P<0.05). *Conclusions.* Continuing medical education of primary care physicians has significant role in diagnosis and treatment of patients with IDA. Education programs result in benefits for the patients and physicians.

Red cell and iron 1

0482

ALLELIC DISTRIBUTION AND GEOGRAPHICAL CLUSTERING OF SEC23B MUTATIONS IN CDA II PATIENTS FROM ITALY

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Background. The Congenital Dyserythropoietic Anemia type II (CDA II) is an autosomal recessive disorder characterized by an impaired differentiation-proliferation pathway of the erythroid lineage. The vast majority of CDA II cases are associated with mutations in the SEC23B gene, codifying a component of the cytoplasmic coat protein (COP)II complex, involved in the anterograde transport of correctly folded protein from the endoplasmic reticulum towards the Golgi. Until now, 63 unrelated cases from CDA II International Registry have been described (Schwarz, 2009; Bianchi, 2009; Iolascon, 2009). Among them, 31 cases had Italian origin with 15 different mutations. *Aims.* To characterize the allelic distribution and geographical clustering in Italian country of SEC23B gene mutations. *Methods.* Fifty-four CDA II independent cases from the Italian Registry were included in this study, of which 13 still unpublished. All exons, flanking splice junctions, 5'- and 3'-untranslated regions of the SEC23B gene were amplified to perform mutational screening. Italian country was divided into North, Middle, South in order to assess the geographical clustering and the allelic frequencies of the mutations in each region. *Results.* Overall we found 26 different mutations, of which 11 resulted novel. They included 12 missense, 6 nonsense, 4 frameshift and 4 splice site mutations. The most representative missense mutations were E109K (31%) and R14W (26%): these data substantially confirm the E109K as the most frequent mutation, as observed previously (Iolascon, 2009). Nevertheless, R14W mutation showed an higher frequency when compared to those calculated from International Registry (26% vs 17%). When we analyzed the geographical distribution in Italian country we observed two peaks of elevated frequency for E109K substitution: one in South (42.9%) and another in North (20.8%) Italy. Instead, R14W mutation showed a progressive reduction of frequency from North to South Italy. *Summary/conclusions.* Here, we described 11 novel mutations in CDA II Italian patients. We also characterized the allelic distribution in Italian country of SEC23B gene mutations and we defined the two most frequent mutations in CDA II patients from Italy, confirming previously described data. Moreover, the study of geographical distribution could be useful for the understanding of migration within the Italian peninsula.

0483

IN VITRO COMPLEMENT PROTEIN 5 (C5) BLOCKADE RECAPITULATES THE COMPLEMENT PROTEIN 3 (C3) BINDING TO GPI-NEGATIVE ERYTHROCYTES OBSERVED IN PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH) PATIENTS ON ECULIZUMAB

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Background. C5 blockade by the monoclonal antibody eculizumab produces significant clinical improvement in most PNH patients by preventing intravascular hemolysis. However, in almost all PNH patients on eculizumab a proportion of GPI-negative (GPI-neg) red blood cells (RBC) become coated with C3 ($C3^+$), and thus potential prey to phagocytosis by macrophages. In most cases this phenomenon has relatively little clinical relevance; however, in some patients it may limit the clinical benefit from eculizumab. *Aims.* To investigate mechanism and modalities of C3 binding to RBCs of PNH patients on eculizumab. *Methods*. We have set up an *in vitro* model of the above *in vivo* phenomenon by incubating RBCs from PNH patients with AB0-compatible sera from either healthy subjects or PNH patients on eculizumab. In these sera complement alternative pathway is activated by either acidification or spontaneous 'tick-over' only. At serial time points C3 binding was assessed by flow cytometry. *Results.* In untreated PNH patients there was no evidence of C3 binding on either normal or GPI-neg RBCs (Figure 1A). The same was true when RBCs from untreated PNH patients (n=11) were incubated with acidified sera from healthy subjects. However, when RBCs from untreated PNH patients were incubated with acidified sera from patients on eculizumab, we observed in

every case the appearance of a population of C3+ GPI-neg RBCs (Figure 1B). Just as in PNH patients on eculizumab, this population coexists with a population of GPI-neg RBCs without C3 and with a population of normal RBCs, none of which became C3+. The percentage of C3+ GPI-neg RBCs increased with time from 6±2% at 1 hour to 64±15% at 24 hours. In the presence of 1.25 mM MgCl2 the proportion of C3+ GPI-neg RBCs increased further, in some cases up to nearly 100%. Also with RBCs from PNH patients on eculizumab upon incubation with acidified sera virtually all GPI-neg RBCs eventually became C3+. Serum acidification *in vitro* produces discrete GPI-neg RBCs hemolysis (20-70%) whereas intravascular hemolysis, usually, is not observed *in vivo* in PNH patients on eculizumab. In order to mimic more physiological conditions, we next investigated the effects of spontaneous complement activation. Under these conditions, not surprisingly, hemolysis of GPI-neg RBCs in sera from patients on eculizumab was negligible (0-10% at 5 days); however, even under these very mild complement activation conditions, C3 binding on GPI-neg RBCs was still substantial (Figure 1C): 20±18% at 3 days and 29±23% at 5 days. *Conclusions.* We have reproduced *in vitro* the C3 binding on GPI-neg RBCs observed in PNH patients on eculizumab. The *in vitro* kinetics of C3 binding suggests that the two populations of GPI-neg RBCs (with and without C3 binding), observed both *in vivo* and *in vitro*, do not result from intrinsically different properties of RBCs, but rather from how long they have been exposed to activated complement. A discrete population of C3+ GPI-neg RBCs is produced only when C5 is blocked by eculizumab, strongly supporting the notion that it results from a specific interaction between eculizumab C5 blockade and GPI-neg RBCs.

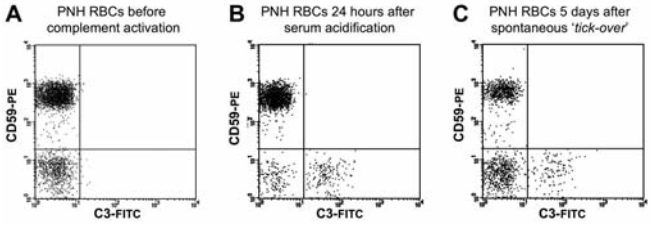

Figure 1. C3 binding on PNH red blood cells after in vitro C5 blockade.

0484

RIBOSOMAL DEFICIENCIES CAUSE TRANSLATIONAL DEREGULATION OF GENES CRUCIAL FOR ERYTHROPOIESIS

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Background. Diamond Blackfan Anemia (DBA) is a rare congenital pure red cell aplasia in which mutations in distinct ribosomal proteins (RPs) for small (40S) and large (60S) subunit have been identified in over 54% of patients. It remains elusive why loss of such ubiquitously expressed proteins causes a distinct erythroid phenotype. *Aims.* Previously, we showed that selective translation of transcripts with a complex RNA structure in the 5'untranslated region (5'UTR) is crucial in Stem Cell Factor (SCF) dependent expansion of the early erythroid compartment. Transcripts such as Immunoglobulin binding protein 1 (Igbp1) are actively recruited into translating polyribosomes upon SCF signaling which activates the PI3K-mTOR pathway leading to release of eukaryote initiation factor 4E (eIF4E), a limiting factor for assembly of the scanning complex. We therefore investigated if polysome recruitment of specific mRNAs is affected in erythroblasts deficient for RPs. *Methods.* Mouse primary erythroblasts derived from p53-deficient and wild-type (wt) fetal livers were cultured in presence of erythropoietin, SCF and dexamethasone under serum free conditions. Knockdown of RPs was achieved using lentivirus-delivered shRNA followed by analysis of subpolysomal and polysome-bound RNA by sucrose gradient centrifugation. Subpolysomal and polysome-bound RNA fractions from 3 independent experiments were used for expression profiling. The ratio of polysome association was calculated per gene for each experiment and datasets from wt, scrambled shRNA treated, Rps19 and Rpl11 deficient conditions were compared using F-test with random variance model (P<0.0005). *Results.* Downregulation of either Rps19 or Rpl11 resulted in reduced proliferation and inhibition of differentiation in comparison to control cells. Cells showed a specific reduction of the 40S and 60S ribosomal subunit upon knock down of Rps19 and Rpl11, respectively. Ratios comparisons revealed that SCF-dependent transcripts were found enriched in polyribosomes, whereas a set of mRNAs, including Fxc1 (fractured callus expressed-1), Bag-1 (Bcl2-associated athanogene 1), Csde1 (cold shock domain containing E1) and Cdc25B (cell division cycle homolog B) were lost from translating polyribosomes upon RP deficiency. We next analyzed selective translation of some of these genes in erythroblasts from DBA patients. Erythroblasts could be cultured from peripheral blood of DBA patients although expansion potential of the cultures was decreased compared to control cultures. Protein expression of identified target genes (CSDE1, CDC25B) was down regulated in erythroblasts derived from DBA patients as compared to controls, whereas their transcript level on total mRNA remained unchanged, indicating a specific translation defect. Further investigation showed that loss of Csde1 perturbed cell cycle progression and erythroid differentiation. *Summary.* In conclusion, we identified transcripts whose translation is selectively affected in RP deficient erythroblasts cultured from mouse fetal livers or DBA patients. We propose that the erythroid phenotype in DBA patients is caused by defective translation of a specific set of mRNAs that are essential for erythroid development.

0485

THE SFLT-1 TO PLGF RATIO IS IMPAIRED IN PATIENTS WITH THALASSEMIA AND SICKLE CELL DISEASE

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Background. Placental growth factor (PlGF) is a member of the vascular endothelial growth factor family and is associated with inflammation and with pathologic angiogenesis. PlGF is released from marrow erythroid cells. Serum PlGF concentrations have been reported to be elevated in patients with sickle cell disease. The fms-like tyrosine kinase-1 or sFlt-1 is a soluble form of vascular endothelial growth factor receptor-1 (VEGF-R1), which binds to and sequesters circulating free vascular endothelial growth factor (VEGF) and free PlGF, thereby neutralizing their pro-angiogenic effects. PlGF and VEGFR-1 are key molecules in regulating the angiogenic switch during pathological conditions maintaining the proangio-and anti-angiogenic balance. Perturbations of this balance leads to various degree of endothelial dysfunction, a common pathology in patients with hemoglobinopathies and specifically induce pulmonary hypertension (PHT). In this study we assessed the PlGF and sFlt-1 levels in patients with hemoglobinopathies at steady state. *Patients and Methods.* One hundred twenty three patients with hemoglobinopathies and 20 apparently healthy individuals were included in the study divided in groups as follows: Group A: 38 patients with thalassemia intermedia; Group B: 41 patients with transfusion-dependent beta-thalassemia; Group C: 33 patients with beta-thalassemia/sickle cell disease (SCD) Group D: 11 patients with beta-thallassemia and clinical evidence of PHT, and; Group E: 20 individuals served as control group In patients and controls we performed measurements of PlGF and sFlt-1 with fully automated electrochemiluminescence assays using the immunochemistry autoanalyzer Roche cobas e411. This method provides rapid and reliable assessments and follow-up of patients any time in the day compared to immunoenzymatic assays. *Results.* We found that: a) serum PlGF levels were increased in all groups of patients compared to controls (42.7±19.9 pg/mL, 49.9±23.4 pg/mL, 27.5±9.4 pg/mL, 58.1±27.6 pg/mL for group A, B, C and D, respectively vs 17.1±3.9 pg/mL for control group, P<0.001), b) serum sFlt-1 levels were also increased in all groups of patients compared to controls (89.3±18.3 pg/mL, 90.2±24.0 pg/mL, 89.4±15.3 pg/mL, 117.2±43.5 pg/mL, respectively vs 76.7±11.1 pg/mL, P<0.001 for control group and c) sFlt-1 to PlGF ratio was decreased in all groups of patients compared to controls (2.4±0.9, 2.2±1.2, 3.5±1.0, 2.2±0.8, respectively vs 4.7±1.1 for control group, P<0.001. The most significant changes were found in patients with PHT. *Conclusions.* These findings indicate that patients with thalassemia syndromes and SCD appear to have increased degree of angiogenesis, which is more pronounced in patients with thalassemia compared to patients with SCD and markedly increased in patients with PHT. The decreased sFlt-1/PlGF ratio in almost all patients suggest that the proangio-and anti-angiogenic system is shifted towards to proangiogenic state, providing evidence that patients with hemoglobinopathies even in the steady phase have altered angiogenic state and

low-grade inflammation. The results are in accordance to those published earlier for SCD (Blood, 2008;112:856-865.), where the authors demonstrated that PlGF levels were intrinsically elevated due to the increased red cell turnover and may contribute to inflammation and PHT seen in the disease.

0486

PREFERENTIAL PATTERNS OF MYOCARDIAL IRON OVERLOAD BY MULTISLICE MULTIECHO T2* CMR IN THALASSEMIA MAJOR PATIENTS

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Background. T2* multislice multiecho cardiac magnetic resonance (CMR) technique has permitted quantification of myocardial iron overload (MIO) by a segmental approach. Little is known in the literature about patterns of iron store in hemochromatosis. *Aims.* Our study aimed to investigate myocardial iron overload in thalassemia major (TM) patients by segmental T2* CMR technique, in order to determine if there were preferential patterns of iron deposit. *Methods.* Five hundred and nineteen TM patients underwent CMR. Three parallel short-axis views (basal, medium, and apical) of the left ventricle were obtained by a T2* gradient-echo multiecho sequence. The images were analyzed using a previously validated, custom-written software (HIPPO-MIOT IFC-CNR®). The myocardium was automatically segmented into a 16-segment standardized LV model and the T2* value on each segment was calculated. The global T2* value averaged over all 16 segmental T2* values, as well as the mean values over the basal, middle, and apical slices were automatically provided. The T2* value in the mid-ventricular septum was evaluated by averaging the T2* values in segments 8 and 9. Four different main circumferential regions were defined by averaging the corresponding segmental T2* values: anterior (segments 1,7,13), septal (segments 2,3,8,9,14), inferior (segments 4,10,15) and lateral (segments 5,6,11,12,16). *Results.* Two-hundred and twenty-nine patients showed global T2* value < 26 ms, corresponding to significant global heart iron overload [4]. The analysis was focused on this patient population, subsequently divided into two groups: severe iron overload (N = 83, global T2* < 10 ms) and mild-moderate iron overload (N =146, global T2* between 10 and 26 ms). For each group, segments were sorted by mean T2* value. Segment order was significantly preserved between the two groups (r = 0.91, P<0.0001). Significant circumferential variability was found in patients with overall heart iron overload as well as in both groups (P<0.0001). The mean T2* value over the anterior region was significantly lower than the mean T2* values over the other regions and the mean T2* over the inferior region was significantly lower than the T2* values over the septal and lateral regions. This pattern was preserved within each single slice. We found a significantly higher T2* value in the basal slice versus the medium and apical slices in patients with severe iron overload. *Conclusions.* A preferential pattern of iron store in anterior and inferior regions appears to be present in TM patients with severe and mild-moderate iron overload. The preserved pattern between the groups prevents attributing this datum to additive susceptibility artefacts, which are negligible in heavily iron-loaded patients. A segmental T2* CMR approach could identify early iron deposit, useful for tailoring chelation therapy and preventing myocardial dysfunction in the clinical setting.

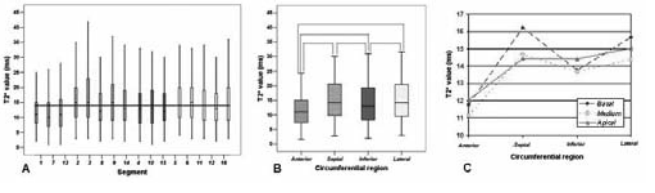

Figure 1. CMR data in patients with global heart T2* <26 ms.

0487

CORRELATIONS BETWEEN PANCREATIC IRON BURDEN AND HEART IRON OVERLOAD AND FUNCTION BY MRI IN A LARGE COHORT OF THALASSEMIA MAJOR PATIENTS

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Background. The leading cause of death in thalassemia major (TM) is heart failure, but impairment of the pancreatic function is common. Multiecho T2* magnetic resonance imaging (MRI) is a well established technique for heart and liver iron overload assessment. *Aims.* Aim of our study was to investigate using quantitative MRI the correlation between heart iron overload and function with pancreatic siderosis in TM patients. *Methods.* We studied 147 TM patients (233 males, age 31±9 years) enrolled in the Myocardial Iron Overload in Thalassemia network. Myocardial iron overload was measured by T2* multislice multiecho technique. Biventricular function parameters were quantitatively evaluated by cine images. Pancreatic iron burden was measured using a T2* gradient-echo multiecho sequence. *Results.* Significant positive correlations were found between the pancreatic T2* values and the global heart T2* values (r=0.44, P<0.0001) and the number of segments with normal T2*. A normal pancreatic T2* value (≥26 ms) showed a negative predictive value of 100% for cardiac iron. Pancreatic T2* values were positively related with left ventricular (r=0.21, P=0.028) (Fig) and right ventricular (r=0.23, P=0.015) ejection fractions. *Conclusions.* Pancreatic iron overload is positively correlated to myocardial iron overload and negatively correlated to bi-ventricular systolic function. Pancreas T2* had a powerful predictor for heart iron burden. Thus, staging abdominal and cardiac MRI could significantly reduce need for sedation in young patients, costs and magnet time, particularly in countries where the cardiac MRI availability is difficult.

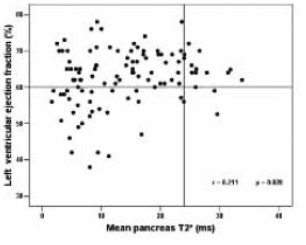

Figure 1.

0488

CHRONIC COLD AGGLUTININ DISEASE: HIGH RESPONSE RATES AND DURABLE REMISSIONS AFTER FLUDARABINE AND RITUXIMAB COMBINATION THERAPY

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Background. Chronic cold agglutinin disease (CAD) is a subtype of cold-antibody autoimmune hemolytic anemia. A clonal lymphoproliferative bone marrow disorder is present in most, if not all patients traditionally classified as having 'primary' CAD. Treatment with rituximab is the only established drug therapy, leading to partial response (PR) in 45-60% of the patients, while complete responses (CR) are rare. The median response duration is only 11 months. *Aim.* We wanted to improve on these results by studying the potential of fludarabine and rituximab combination therapy, and we present here the preliminary findings. *Method.* We performed a prospective, uncontrolled multi-center trial. After informed consent had been obtained, eligible patients received infusions of rituximab, 375 mg/m^2 day 1, 29, 57 and 85; and fludarabine orally, 40 mg/m^2 day 1-5, 29-34, 57-51 and 85-89. Responses were assessed according to previously published, strict definitions, and adverse events were recorded. *Results.* 29 patients were treated, 12 men and 17 women, with a median age of 73 years (range, 39-87). Median hemoglobin level was 8.7 g/dL (range, 5.4-15.6; two non-anemic patients being treated because of disabling circulatory symptoms).

Median IgM concentration was 2.8 g/L (0.94-19.2); cold agglutinin titer, 1024 (64-64000); and cellular kappa/lambda ratio in bone marrow aspirates, 8.4 (3.5-800). Lymphoplasmacytic lymphoma or marginal zone lymphoma was found in bone marrow biopsy specimens in 59%. Following therapy, we observed an overall response rate of 77%, including 5 CR (19% of evaluable patients) and 15 (58%) PR. Six patients (23%) were non-responders, while response evaluation is still pending in 3. Among 9 patients who had previously received rituximab single agent therapy without response, 1 achieved CR and 4 PR following the combination therapy. Median time to response was 4 months (1-6). Median increase in hemoglobin level was 2.5 g/dL (-1.2 - +5.7) in the total cohort and 3.0 g/dL among the responders. Serum IgM concentrations decreased by median 66% of pretreatment levels. At a median follow-up of 30 months (range, 2-66) after achieving remission, the median and even the lower tertile of response duration has not yet been reached (range, 6 - >66 months). Grade III or IV toxicity occurred in 11 patients (38%) including grade IV adverse effects in 5 (17%). *Conclusions.* Fludarabine and rituximab combination therapy is very efficient in patients with CAD, leading to high response rates as well as durable remissions. For the first time, we report a considerable number of CRs after therapy for CAD; and remissions have been achieved in patients who did not respond to previous single agent therapy with rituximab. Toxicity is more prevalent as compared to rituximab monotherapy, and we have not studied the long-term effects of fludarabine in this patient group. Some caution should probably be exerted in the youngest as well as the oldest and most frail patients. It remains to be established whether the fludarabine-rituximab combination should be considered first-line or an efficient second-line therapy in patients with CAD requiring pharmacologic treatment.

0489

HYPOXIA CAUSES SIGNIFICANT AUTONOMIC DYSREGULATION AND SIGHS CAUSE VASO CONSTRICTION IN PATIENTS WTIH SICKLE CELL ANEMIA: A POSSIBLE LINK BETWEEN PULMONARY PATHOLOGY AND VASO OCCLUSIVE CRISIS

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Background. Nighttime hypoxia predicts stroke and crisis frequency in subjects with sickle cell disease (SCD) suggesting a role for sleep disordered breathing and recurrent hypoxia in the pathophysiology of SCD. *Aims.* Determine the mechanism by which transient hypoxia modulates microvascular blood flow in subjects with SCD. *Methods.* Using an ethics committee approved protocol; we established a method to induce a single episode of hypoxia that mimicked those occurring naturally during sleep. To accomplish this, we exposed normal and SCD subjects who had continual monitoring of electrocardiogram, tidal volume, oxygen saturation, and microvascular blood flow to five breaths of pure nitrogen. An autoregressive model was used to examine autonomic balance derived from heart rate variability (HRV) measurements and we used standard statistical methods to study the relation of sighs seen in the tidal volume tracing (Vt) to episodes of marked decrease in microvascular perfusion. The high frequency component of the HRV signal reflects parasympathetic nervous system tone. *Results.* 8 SCD subjects and 9 normal controls were studied. In a total of 27 experiments in SCD subjects with two hypoxic exposures, there were no severe adverse events. The nadir of the SaO2 was 80% of baseline in SCD and 85% in normals. Microvascular blood flow was measured by laser-Doppler flowmetry and did not decrease in response to the hypoxia, as we had expected. However, we did note a significant change in the heart rate in SCD subjects that was not seen in normals. We also noted periodic transient episodes of marked vaso-constriction commonly in SCD subjects and rarely in normals. Because of the heart rate changes we examined respiratory corrected heart rate variability and found significant loss of the parasympathetic component of the cardiac autonomic balance in response to hypoxia in SCD but not in normal subjects. The periodic drops in perfusion were associated with deeper breaths (sighs) seen in the tidal volume recording. The odds ratio for a sigh causing vasoconstriction was 1750 P<.001 indicating that sighs induce vaso constriction in SCD and normals. However, there is a 77% probability that a sigh will cause vaso constriction in SCD compared to only 14% in normal subjects (P<.008). *Summary.* Subjects with SCD have significant loss of heart rate variability in response to transient hypoxia indicating severe autonomic deregulation. Furthermore, sighs cause transient neurally- mediated vaso constriction and this effect is significantly enhanced in SCD subjects. Vaso constriction results in retention of red cells in the microvasculature increasing the likelihood that sickling will occur before the rbc exit into larger vessels. Thus the rigid SRBC become lodged in the microvascularture resulting in vasoocclusion. These data suggest that the association of sickle crisis with sleep disordered breathing and asthma may be mediated through a mechanically sensed autonomic /neural transmitted vaso-constriction leading to SRBC retention in the microvasculature and subsequent vaso-occlusion rather than by a direct effect of hypoxia on HbS as the initiating event in vaso-occlusion.

0490

A NOVEL MUTATION IN INTESTINAL ISOFORM OF DIVALENT METAL TRANSPORTER 1 (DMT1-1A)

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Background. Divalent metal transporter 1 (DMT1) mediates apical iron uptake into duodenal enterocytes and also transfers iron from the endosome into the cytosol after cellular uptake via the transferrin receptor. Mutations in DMT1 cause systemic iron deficiency and microcytic anemia. The three patients with DMT1 mutations described so far developed highly transferrin saturation and mildly elevated serum ferritin, despite liver iron overload. Anemia is present from the birth. The molecular defect is common to intestinal and erythroid isoforms, which could explain the elevated transferrin saturation and the microcytosis. Four DMT1 isoforms exist resulting from mRNA transcripts that vary both at their 5' ends (starting in exon 1A or exon 1B) and at their 3' ends giving rise to mRNAs containing (+) or lacking (-) the 3' IRE (iron-responsive element). Exon 1A is located 1.9 kb upstream of the first exon ("1B") of the previously characterized human DMT1 and is followed by a consensus splice sequence. The exon 1A is spliced directly to exon 2. The 1B isoform is ubiquitous, whereas the 1A isoform is tissue-specific, predominantly in the duodenum and kidney. Here, we report a novel DMT1 variant with a mutation in 1A isoform due to A>T transition at nucleotide position 652 of Ensembl sequence ENSG00000110911. *Case Report.*, This 55-'years-'old Caucasian female with unrelated parents was admitted to our hospital for mild anemia and microcytosis (Figure 1). We observed a mild hypochromic microcytic anemia with a hemoglobin level of 9 g/dL, a mean cell volume (MCV) of 56 fL, and a mean hemoglobin concentration (MCH) of 30.9 pg, the reticulocyte count was $69\times10^3/\mu L$. Transferrin saturation was elevated (129%) as well as ferritin levels and soluble transferrin receptor values. There were no alterations in hemoglobin electrophoresis and there was no enzymatic defect. *Results.* Sequencing of the exons and exon-intron boundaries of the DMT1 gene revealed the proband compound heterozygote for c.GGC723>GTC, also described in literature, and a novel A>T substitution in intron 1A at position 1 following the acceptor splice site. The latter could lead to splicing alterations as verify by in silico and *in vitro* tools and not affect the mRNA expression of DMT1. *Conclusions.*The DMT1 1A mutation has an homozygote expression in enterocytes, otherwise there is no expression of this isoform in hepatic or erythroid cells. Compound heterozygote condition is present only in enterocytes, where we hyphotize that the exon 8 G>T transition causes a functional alteration while mutation in DMT1 1A causes a reduction of protein expression.

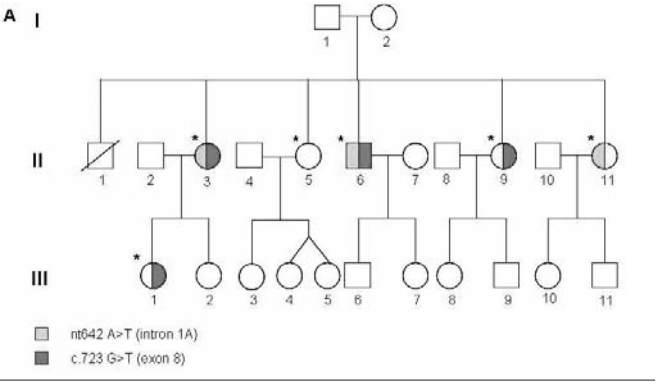

Figure 1.

0491

EXPRESSION OF MICRORNAS IN RETICULOCYTES FROM BETA THALASSEMIA INTERMEDIA PATIENTS

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MicroRNAs (miRNAs) represent a novel class of endogenous non-coding RNAs of approximately 20-22 nucleotides. In mammalian cells, miRNAs negatively regulate gene expression by inhibiting translation of target RNAs or degrading messenger RNAs. In normal erythropoiesis, microRNAs play a very important role because they regulate differentiation in most stages. There is an increasing number of studies demonstrating the function of these non-coding RNAs in the pathogenesis and prognosis of erythropoietic disorders, including thalassemias. Beta-Thalassemia (BT) is an inherited hematologic disease caused by reduced or absent synthesis of beta globin chain, leading to a relative excess of alpha globin chains. This imbalance causes hemolysis and impair erythropoiesis. The aim of this study was to analyze the expression profile of miRNAs 24, 144, 155, 210, 221, 222, 223 and 451 in beta-thalassemia intermedia patients. Reticulocytes were separated from peripheral blood samples of control subjects (n≥9) and from untransfused BT IVS-I-6 (T C) homozygous patients without HU therapy (n≥9). Samples from spherocytosis patients with reticulocytosis and synthesis of normal globin chains were also included to confirm miRNA expression profile (data not show). Extraction of miRNA, transcription to cDNAs and Real Time PCR were performed to analyze miRNAs expression, using U47 and RNU6B as endogenous controls. The statistical analyzes showed that the expression levels of the miRNAs 24, 144, 155, 210, 221, 222, 223 and 451 were significantly lower in BT patients compared to control subjects (mean 0.0009 vs 0.193, P=0.030; 0.034 vs 0.357, P=0.04; 0.073 vs 0.736 , P=0.0106; 0.029 vs 0.222, P=0.0244; 0.029 vs 0.556, P=0.0001; 0.048 vs 0.358, P=0.0106; 0.045 vs 0.198, P=0.0056; 0.029 vs 0.169, P=0.0160, respectively). These miRNAs were previously described to act in different stages of normal erythropoiesis. When up-regulated, miRNAs 144 and 451 promotes erythroid maturation and synthesis of hemoglobin, while the miRNA 210 acts in erythroid differentiation. In this study, the low expression of these miRNAs suggests their involving at latest maturation in BT. The interaction between miRNAs 451 and 155 demonstrates that overexpression of miRNA 451 promotes decrease in expression of miRNA 155. However, the data presented in this report shows low expression of both microRNAs. Downregulation of miRNAs 24, 221, 222 and 223 results in erythroid maturation; although this study as shown that the expression profile of these miRNAs was lower in BT, the pathways from which they draw the erythroid differentiation is not clear. This is the first report of miRNAs expression involved in reticulocytes of beta-thalassemia intermedia patients. These results suggest the importance of miRNAs molecular pathways in the BT patient's erythropoiesis which may contribute to the understanding of this pathophysiology.

0492

ACE-536, A MODIFIED TYPE II ACTIVIN RECEPTOR INCREASES RED BLOOD CELLS IN VIVO BY PROMOTING MATURATION OF LATE STAGE ERYTHROBLASTS

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Anemia is a common debilitating complication associated with diseases such as chronic kidney disease and cancer chemotherapy. Currently, administration of recombinant erythropoietin (EPO) represents the most common treatment for anemia. However, a significant number of people remain hypo or non-responsive to EPO treatment. Several members of the TGFβ super family have demonstrated a role in erythropoiesis. We developed a soluble receptor Fc fusion chimera of a modified form of activin receptor IIB (ACE-536) and studied its effect on erythropoiesis. *In vivo* subcutaneous administration of ACE-536 (10 mg/kg subcutaneously, twice weekly) in C57BL/6 mice twice weekly resulted in a significant increase in hematocrit, hemoglobin and red blood cells (RBC count) compared to a vehicle group after 4 days, although these parameters were already significantly increased 24 hours post treatment. These observations were evident in the presence of an EPO neutralizing antibody suggesting that EPO is not directing the initial RBC response. No changes in red blood cell half life were noted in mice treated with ACE-536. Differentiation profiling of bone marrow and Splenic erythroblasts by flow cytometric analysis revealed that ACE-536 promotes faster maturation of erythroblasts. ACE-536 treatment for 72 hours resulted in a decrease in basophilic erythroblasts and an increase in subsequent late stage poly, ortho chromatophilic and reticulocytes in bone marrow and spleen compared to the TBS treated mice. ACE-536 (10 mg/kg) treatment of Sprague Dawley rats (N=4) increased the rate of reticulocyte formation compared to the vehicle group, but did not alter the rate of reticulocyte maturation in the peripheral blood. Furthermore, ACE-536 (10 mg/kg) treatment together with recombinant human EPO (1800 units/kg) for 72 hours increased RBC, hematocrit and hemoglobin by 23% over TBS treated vehicle group and significantly enhanced the effects compared to EPO treatment alone. Consistent with its role in proliferation, EPO treatment increased the splenic basophilic erythroblast formation. However, ACE-536 treatment with EPO significantly promoted maturation of basophilic erythroblasts demonstrating a novel mechanism during erythroid differentiation. This novel mechanism of ACE-536 offers the opportunity for a new treatment for anemia with a safety and efficacy profile that may be distinct from EPO.

0493

EVALUATION OF ERYTHROPOIESIS AND IRON METABOLISM IN RESPONSE TO ERYTHROPOIETIN INJECTIONS IN A MOUSE MODEL OF GENERALIZED INFLAMMATION AND ANEMIA

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Background. Several factors contribute to the development of anemia of chronic disease (ACD), such as an impaired proliferation of erythroid progenitors, reduced red blood cell (RBC) half life, a blunted erythropoietin response and dysregulation of iron homeostasis. Pro-inflammatory cytokines play a major role in the onset of anemia. In mouse, acute anemia stimulates BMP4 expression in the spleen and induces the proliferation of BMP4-responsive stress erythroid progenitors allowing rapid recovery from anemia. The effect of inflammation on this stress erythropoiesis is not known. *Aims.* to explore erythropoiesis and iron metabolism in a mouse model of chronic inflammation and to assess the efficacy of Epo injections in correcting the ACD. *Methods.* Mice (C57BL/6) received a single intraperitoneal injection of Zymosan at day 1 (Z1) and were analyzed at Z2, Z5, Z12 and Z17. Epo injections in inflammed mice were performed on four consecutive days starting at Z5. Double Ter119/CD71 labelling was used to analyze erythroblast differentiation by FACS, in bone marrow and spleen. Spleen BMP4 expression was followed by RT-qPCR and immunohistochemistry. Haematological parameters were recorded and RBCs survival was evaluated. Serum hepcidin concentrations and macrophage ferroportin expression were assessed. *Results.* Zymosan-induced inflammation lead to a long-lasting anemia with reduced number of RBCs and low Hb levels (10 g/dL). We found that the Zymosan injection shortened RBCs survival. Furthermore, bone marrow erythropoiesis was fully suppressed with an increase in the percentage of apoptotic $Ter119^+$ cells and it did not respond to Epo injections. In the spleen, inflammation only moderately stimulated the percentage of erythroblasts. However, Epo injections led to a 10-fold increase in immature erythroblasts one day after the final Epo injection, followed three days later by a similar increase in the proportion of mature erythroblasts. Epo also induced a significant increase in BMP4 expression in spleen macrophages. Serum hepcidin levels were dramatically elevated in inflammed mice and ferroportin expression in spleen macrophages was repressed. However, Epo injections decreased hepcidin concentrations but the response was delayed as compared to control mice. Ferroportin expression increased concomitantly. Nine days after the final injection, Epo had allowed a partial correction of the anemia(Hb=11.2 g/dL as compared to 14 g/dL in controls). *Conclusions.* These results highlight the differences between bone marrow and spleen erythropoiesis. They also show that Epo injections in mouse can partially correct the ACD by stimulating spleen stress erythropoiesis and progressively decreasing hepcidin expression and suggest that Epo favours iron mobilization from tissue iron stores, despite the presence of inflammation.

0494

ANALYSIS OF SEVEN RIBOSOMAL PROTEIN GENES IN ITALIAN DIAMOND BLACKFAN ANEMIA PATIENTS

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Background. Diamond Blackfan anemia (DBA; MIM 205900) is a rare, pure red cell aplasia of childhood due to an intrinsic defect in erythropoietic progenitors. Somatic malformations are present in about 40% of patients. For about 10 years, RPS19 was the only gene found mutated in DBA patients and its mutations accounted for 25% of cases. In the last four years, however, heterozygous mutations in several genes (RPS24, RPS17, RPL35A, RPL5, RPL11, RPS7) encoding for ribosomal proteins (RP) of either the small or the large ribosomal subunit have been reported. Recently, Doherty et al. (Am J Hum Genet, 2010), described the involvement of two other genes, RPS10 and RPS26, in about 10% of DBA patients.*Aims.* To characterize 135 Italian DBA patients for mutations in seven genes, all encoding for RPs. *Methods*. We sequenced exons, intron-exon boundaries of RPS19 in 135 Italian unrelated DBA patients and RPS24, RPL35A, RPL5, RPL11 genes in 96 patients with no mutations in RPS19. So far RPS26 and RPS10 genes have been sequenced in 23/68 patients not mutated in other RP genes; sequencing of these two genes in the remaining 45 is ongoing. One hundred normal chromosomes were studied for each identified DNA change to rule out polymorphisms. *Results.* The analysis of In the Italian DBA cohort demonstrated 39/135 mutations in RPS19 gene (29%), 14/135 (10%) and 12/135 (9%) mutations in RPL5 and RPL11 genes. Only 2/135 (1.5%) patients showed mutations in RPS24. No mutations were found in RPL35A. Regarding the last two genes identified, we found 3 heterozygous mutations in RPS26 and one in RPS10. The first one is a missense mutation (c.3G>A) involving the first ATG codon. The second mutation is a deletion of 4 bp in exon 3 with the creation of a premature stop codon and the third is a mutation at donor splice site of intron 1. A missense mutation with substitution of Treonine with Isoleucine at codon 138 was found in RPS10 gene. All mutations but one were new mutations (i.e. not yet described in the literature). All RPS10 and RPS26 mutations were *de novo*. No malformation was present in these patients. All RPS26 mutated patients were unresponsive to steroid therapy and transfusion dependent. *Conclusions.* Overall, mutations in 5 RP genes (RPS19, RPL5, RPL11, RPS10 and RPS26) account for more than 50% of all DBA cases. RPL5 and RPL11 are associated with hand and cleft malformations. No correlation was found between somatic malformations and RPS26 mutations. We observed a severe phenotype in term of transfusion dependence among RPS26 patients.

0495

ERYTHROPHAGOCYTOSIS BY ANGIOGENIC ENDOTHELIAL CELLS IS ENHANCED BY LOSS OF ERYTHROCYTE DEFORMABILITY

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Objective. Angiogenic endothelial cells can function as phagocytes and phagocytosis is initiated via the opsonin lactadherin. In this study, we examined the interaction between lactadherin-opsonized erythrocytes with reduced deformability and angiogenic endothelium, as loss of deformability is characteristic for suicidal and aged erythrocytes. *Methods.* We used the RGD-modified erythrocyte model and investigated the deformability parameter by cross-linking erythrocyte membranes, through treatment with glutaraldehyde (GA). Association *in vitro* with primary endothelial cells was detected by flow cytometry and visualized by light, fluorescent and electron microscopy. Involvement of two crucial factors in phagocytosis, αv-integrins and Rho GTPase family member Rac1 was studied using siRNA technology. Modified erythrocytes were administered *in vivo* into tumor-bearing mice to detect phagocytosis by endothelial cells. *Results.* GA-treated (rigid) RGD-modified erythrocytes showed a strongly enhanced endothelial cell association compared to flexible RGD-modified erythrocytes. Knockdown by siRNA lipoplexes of αv-integrins and Rac1, confirmed classical tethering and internalization of rigid RGD-erythrocytes. Upon *in vivo* administration, tumor endothelium showed pronounced erythrophagocytosis. *Conclusion.* The pronounced phagocytosis of opsonized erythrocytes with reduced deformability by angiogenic growth factor-activated endothelial cells (Figure 1), evoke new insights in endothelial cell function and suggest a role for these endothelial cells in (haematological) disorders because of their capacity to clear disordered erythrocytes.

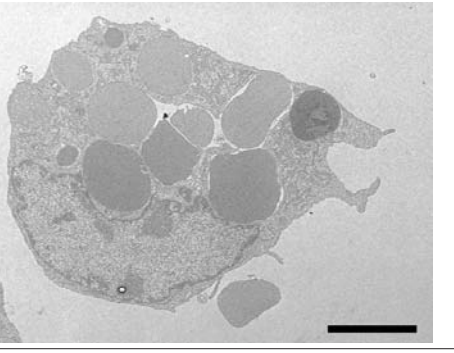

Figure 1 Pronounced erythrophagocytosis by HUVEC.

0496

MYOCARDIAL FIBROSIS BY DELAYED ENHANCEMENT CARDIOVASCULAR MAGNETIC RESONANCE AND HCV INFECTION IN THALASSEMIA MAJOR PATIENTS

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Background. Delayed enhancement (DE) cardiac magnetic resonance (CMR) technique is the only validated non-invasive approach for detecting myocardial fibrosis. In thalassemia major (TM), myocardial fibrosis has been detected using the DE technique and a positive correlation with anti-HCV antibodies has been described. However, HCV-induced cardiomyopathy is still controversial. *Aims.* The aim of our study was to verify a possible correlation between myocardial fibrosis detected by DE CMR and chronic HCV infection in a large retrospective cohort of TM patients. *Methods.* We analysed 434 TM patients (233 males, mean age 31±9 years) consecutively enrolled in the MIOT (Myocardial Iron Overload in Thalassemia) study. DE images were acquired to detect myocardial fibrosis. HCV-RNA tests were sensitive to detect more than 50 copies/mL. *Results.* Ninety out of 434 TM patients (21%) were found to have myocardial fibrosis by DE CMR technique. There was a significant correlation between the presence of myocardial fibrosis and the HCV-RNA positive patients plus the patients with a previous diagnosis of chronic hepatitis C (CHC) treated by alpha-interferon (P=0.026). *Conclusions.* Our finding supports the hypothesis that HCV infection can be involved in the pathogenesis of myocardial fibrosis in the multitransfused TM patients, who could therefore benefit from therapeutic interventions directed towards the eradication of HCV.

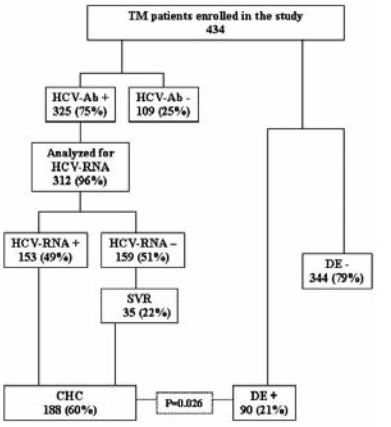

Figure.

0497

EVALUATION OF TINF2 AND SBDS GENE MUTATIONS AND SCREENING TELOMERE LENGTH IN ADULT JAPANESE PATIENTS WITH ACQUIRED BONE MARROW FAILURE SYNDROMES

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Background and *Aims.* We have recently shown that some patients with cryptic and late on set Dyskeratosis Congenita (DKC) patients among those with acquired aplastic anemia (AA) or myelodysplasia syndrome (MDS) have short telomeres due to mutation in the telomerase gene component TERC or TERT. In addition, several recent studies showed heterozygous mutations of the telomere maintenance associated genes TINF2 and SBDS in 1-5% of patients with acquired AA. The largest controlled epidemiologic study reported that the incidence of AA in the West is 2 per million per year and is about 2- to 3-fold higher in Asia. The analysis of the TINF2 and SBDS genes among adult Asian populations with AA and MDS, to the best of our knowledge, had never been done. Therefore, we carried out an investigation to determine whether mutations in these genes can be found in adult Japanese patients with acquired BMFS. Clinically it is very important to identify these patients with shortened telomeres and pathogenic mutations of telomere associated genes, because these patients will likely exhibit refractoriness to conventional immunosuppressive therapy (IST). Moreover, previous report revealed that non-responders of IST showed shorter telomeres than responders in AA patients. Therefore, in this study, we also evaluated telomere length in peripheral blood from patients at the time of diagnosis with AA to clarify its usefulness in treatment strategies. *Methods.* We screened the entire coding region of the SBDS gene and exon 6 of TINF2 by direct sequence. We used Southern blot analysis to measure the length of telomeres in peripheral blood from AA and BMF patients with mutations in telomere associated genes. *Results.* Among 120 Japanese patients with acquired bone marrow failure syndromes (BMFS), we identified two patients (1.7%) with TINF2 heterozygous mutations (P283H and the novel n865-866 dinucleotide CC deletion), and one patient (0.8%) with SBDS heterozygous mutation (intervening sequence (IVS)+2T/C mutation at intron 2). Both patients with TINF2 mutation show extremely short telomeres as compared to healthy age-matched controls, but the patient with the SBDS mutation did not show obvious short telomere. These patients had no evidence of clinical histories and physical anomalies of DKC or Shwachman-Diamond syndrome. The two patients with TINF2 mutations were diagnosed with severe AA, and did not show any clinical response to immunosuppressive therapy. Age-adjusted telomere length did not significantly differ between non-responders of IST (n=10) and responders (n=16) (P=0.488), but those with mutations of telomere associated genes (n=3) were significantly shorter than those without mutations (P=0.017). *Conclusions.* These findings revealed for the first time that mutations in TINF2 and SBDS genes indeed occur in Asian BMFS patients, and screening telomere length is useful for detecting cryptic DKC, but not for predicting responses to IST among BMFS patients.

0498

CONTINUED IMPROVEMENT IN CARDIAC T2* WITH DEFERASIROX TREATMENT OVER 2 YEARS: RESULTS FROM THE EXTENSION OF EPIC CARDIAC SUBSTUDY IN BETA-THALASSAEMIA PATIENTS WITH MYOCARDIAL SIDEROSIS

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Background. The efficacy of chelation in reducing cardiac iron in β-thalassaemia patients has been demonstrated using myocardial T2* in several prospective clinical trials of up to 1 year. Since T2* normalization may take several years, longer-term efficacy and safety assessments are required. In the cardiac substudy of the EPIC trial, patients have now received deferasirox for up to 2 years in an extension study. *Aims.* To evaluate the efficacy and safety of deferasirox in removing myocardial iron in heavily transfused β-thalassaemia patients over 2 years. *Methods.* Eligible patients were aged ≥10 years with myocardial T2* >5-<20 ms (indicating cardiac siderosis) by cardiovascular magnetic resonance (CMR), left ventricular ejection fraction (LVEF) ≥56%, serum ferritin (SF) >2500 ng/mL, MR (R2) liver iron concentration (LIC) >10 mg Fe/g dry weight (dw), and a lifetime minimum of 50 transfused blood units. Deferasirox was initiated at 30 and increased to 40 mg/kg/day by the time patients entered the extension. Dose decreases were allowed for safety reasons. Primary endpoint was change in myocardial T2* from baseline to 2 years. *Results.* 101 patients entered the extension study. Baseline myocardial T2* was >5-<10 ms in 39 patients (38.6%) and 10-<20 ms in 62 (61.4%); geometric means were 7.3 ms and 14.6 ms, respectively. Mean deferasirox dose increased from 33.1±3.7 in the core study to 36.1±7.7 mg/kg/day during the extension. After 2 years, myocardial T2* significantly increased from a geometric mean baseline of 11.2 to 15.7 ms (P<0.001). Significant increases also occurred in patients with baseline T2* >5-<10 and 10-<20 ms (Figure).

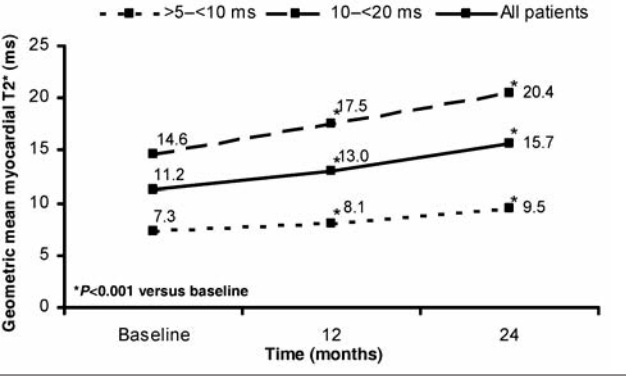

Figure. Geomatric mean myocardial T2* over 2 years.

56.7% of patients with mild-to-moderate baseline cardiac siderosis (10-<20 ms) normalized (≥20 ms) after 2 years; 43% of patients with severe baseline cardiac siderosis (>5-<10 ms) improved to 10-<20 ms. LVEF remained stable. RVEF improved from baseline over 2 years (P<0.001). Both mean LIC (-10.7±12.8 mg Fe/g dw) and median SF (-2343 ng/mL) were reduced significantly from baseline (P<0.001). 86/101 patients (85.2%) completed 2 years. Reasons for discontinuation were: unsatisfactory therapeutic effect (n=7), consent withdrawal (n=4), protocol violation (n=1), lost to follow-up (n=1), abnormal test procedure (decreased T2*; n=1) and abnormal laboratory value (increased urinary protein:creatinine ratio; n=1); no deaths were reported. Investigator-assessed drug-related AEs (≥5%), including increased serum creatinine (SCr), rash and increased alanine aminotransferase (ALT), did not increase in the extension relative to the core study. Three patients dur-

ing the core and one during the extension had increased SCr >33% above baseline and upper limit of normal (ULN) on two consecutive visits. Two patients during the core and two during the extension had increased ALT >10xULN on two consecutive visits; levels were >ULN at baseline in all patients. *Conclusions.* This is the first large prospective study to report 2-year data on cardiac iron removal for any chelator. Continued therapy with deferasirox (30-40 mg/kg/day) removed cardiac iron in β-thalassaemia patients with mild-to-moderate and severe cardiac siderosis and was well tolerated. Normal cardiac function was maintained with decreased hepatic and total body iron burden.

0499

SIMULATION OF THE STRUCTURE, FUNCTION AND MOLECULAR DYNAMICS OF HB S-SÃO PAULO [BETA 6 (A3) GLU TO VAL; BETA 65 (E5) LYS TO GLU] - A NEW VARIANT OF HUMAN HEMOGLOBIN

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Background. Hemoglobin (Hb) S [beta 6 (A3) Glu to Val], present in patients with sickle cell disease, is the most studied hemoglobin structural variant. Concomitant aminoacid replacements that reduce the affinity of the hemoglobin for O2 may lead to desoxi-HbS polymerization and hemolysis to simple carriers. *Aim.* We describe here a new beta chain variant that has a Lysine (Lys) to Glutamic acid (Glu) substitution at position 65 in addition to the Glu to Valine (Val) substitution at position 6. The presence of glutamic acid at position 65 seems to be responsible for the reduced O2 affinity showed by the total hemolysate in absence of organic phosphates. Because of the origin of its carrier, this variant was called HbS-São Paulo. Patient, Methods and *Results.* The proband, an 18-month-old male, had the following hematological parameters: RBC= 3.73×10^6/mm^3, Hb= 8.9 g/dL, Ht= 29.2%, MCV= 78.3 fL, MCH= 23.9 pg, RDW= 18.5%, and reticulocytes= 1.51%. His mother was heterozygous for HbS (AS); his father was not available for studies. Alkaline electrophoresis revealed a band that is faster than Hb A, although acidic electrophoresis, isoelectric focusing and the ID-sickle cell test were compatible with the presence of HbS in heterozygosis. Cation exchange High Performance Liquid Chromatography (HPLC) revealed this anomalous fraction, which had a distinct elution time and represented 29.6% of the total hemoglobin (at the D peak window). Reverse Phase-HPLC showed a mutated beta chain (11.47%), which eluted more slowly than the native beta one. Stability tests were slightly positive, except for the Heinz body test, which was negative. Beta gene sequencing identified two different mutations that were responsible for the Glu to Val and Lys to Glu substitutions; these corresponded to the 6th (GAG to GTG) and 65th (AAG to GAG) codons of the beta chain, respectively. The function of the total stripped hemolysate in the absence and presence of IHP (Inositol Hexaphosphoric acid) was evaluated by the method described by Rossi-Fanelli and Antonini (1958) using a pH between 6.5 and 8.5 and showed a reduced Bohr effect and affinity of hemoglobin for O2 in the stripped state without IHP despite the presence of 12.3% Hb F. To obtain insights into this decreased oxygen affinity, computational models of the double mutant structure were produced from the native structure using the NAMD simulation package and CHARMM parameters, as shown in Figure 1.

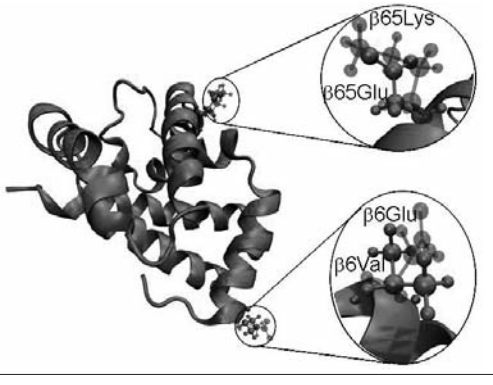

Figure 1. Double mutant of the β-blobin. Structures with transparent representation correspond the native rsidues, which ere sustituted.

Structural analysis revealed that the substitution of Lys65 by a negatively charged glutamic acid may perturb the binding of oxygen by introducing a torsional restraint on the E helix. This torsion seems to impair the entry and exit of oxygen from the heme pocket. *Conclusions.* This event could favor the desoxi-Hb conformation, as demonstrated in the functional tests, and facilitate polymerization of the variant caused by the substitution Glu to Val at position 6 of the beta-chain. *Financial support: FAPESP and CNPq / Brazil.*

0500

CRYPTIC SPLICING SITE USAGE LEADS TO TRUNCATED TMPRSS6 AND IS RESPONSIBLE OF IRON REFRACTORY IRON DEFICIENCY ANAEMIA (IRIDA) IN AN ITALIAN FAMILY

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Background. Iron Refractory Iron Deficiency Anaemia (IRIDA) is an autosomal recessive disease characterised by 1) congenital hypochromic, microcytic anaemia; 2) low MCV (mean corpuscular volume); 3) low serum iron and low transferrin saturation; 4) normal ferritin or ferritin levels in the lower limits of the normal; 5) no response to oral iron treatment and incomplete response to iv iron administration; 6) inappropriately high levels of hepcidin. IRIDA has recently been shown to be caused by mutations in the TMPRSS6 gene, which encodes a type II transmembrane serine protease called matriptase-2 and which is predominantly expressed in the liver. Matriptase-2 (Tmprss6) is the sole protein which can reduce hepcidin expression and hepcidin plays a key role in iron homeostasis. Recently, patients with TMPRSS6 mutations and suffering from IRIDA have been reported. *Aims.* We described a male born in 1955 of Italian origin suffering from IRIDA. He presented microcytic anaemia (64fl) with low serum iron (3 mmol/L) and transferrin saturation (5%) and normal ferritin values (68 mg/L). His hepcidin expression was 9.8 nM (M: 5.8±2.8 nM). This value is inconsistent with the low serum iron of the patient and indicates impaired hepcidin regulation (no hepcidin inhibition in the presence of iron deficiency). This finding reinforced the hypothesis of IRIDA and motivated the sequencing of TMPRSS6. DNA analysis of this patient showed a homozygous G to C mutation in the last nucleotide of exon 15. We made the hypothesis that this mutation is responsible for abnormal splicing. To prove this we analysed matriptase-2 mRNA extracted from peripheral blood cells, since recently it was shown that this protein is expressed at low levels in these cells. *Methods.* DNA was extracted from peripheral blood cells according to standard techniques. All TMPRSS6 coding regions and intron-exon boundaries were sequenced as described by Finberg et al. Nat Genet 2008. mRNA was obtained from peripheral blood cells and converted to cDNA using Superscript II (Invitrogen) and we analysed it for TMPRSS6 expression. A couple of primer in exons 14 and 16 was used to amplify a normal product at 558nt. *Results.* In our patient a smaller aberrant splice transcript was produced. Sequencing analysis showed that another splice donor site was used and led to a novel mRNA with a deletion of 112nt in exon 15. This deletion induces a frameshift leading to a premature stop corresponding to a truncated protein of 595 amino acids instead of the normal one of 811AA. Furthermore this truncated protein has an abnormal carboxy terminus concerning the 16 amino acids before the stop codon. This mutation deletes the serine protease domain of the matriptase-2. *Conclusions.* We have demonstrated that a single mutation in the last nucleotide of exon 15 of the TMPRSS6 gene by activating a cryptic splicing site, is responsible of novel mRNA characterised by a deletion of 112nt. This mutation introduces a frameshift with as a consequence the synthesis of an abnormal protein missing the serine protease domain. This molecular pathology is responsible of IRIDA.

0501

DEVELOPMENT OF LIVER FIBROSIS/CIRRHOSIS IN ADULT HCV POSITIVE THALASSEMIA MAJOR PATIENTS

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Background. It has been shown that iron and HCV infection in patients with thalassemia major (TM) are independent but mutually reinforcing risk factors for the development of liver fibrosis and cirrhosis, with a reciprocal multiplicative effect. *Aim.* The aim of this study was to evaluate the prevalence of HCV infections and iron-related liver disease in

a cohort of adult TM patients in a single care center. *Design and Methods.* We performed a retrospective analysis in a cohort of 132 transfusion dependent TM patients (80 female/52 male, aged 35±6.6 years) followed from 1997 to 2008 (mean 10±3 years) at Hereditary Anemia Center, University of Milan. All patients underwent regular blood transfusions with mean pre-transfusion hemoglobin values 9.3±0.3 g/dL and they were treated with iron chelation therapy. Five patients were lost during follow-up (3 died for liver disease, 2 for cardiovascular disease). To evaluate iron overload we monitored serum ferritin levels. Liver fibrosis was estimated by Transient Elastography (TE); TE thresholds related to Ishak fibrosis score (S) were considered: >7.9 kPa for S≥3; >10.3 kPa for S≥4; >12.0 kPa for S≥ 5. *Results.* Of 132 patients, 111(84%) were anti HCV positive of whom 71(64%) had chronic active hepatitis C because of persistently RNA positive. Liver fibrosis evaluated by TE showed higher values among anti HCV positive patients than negative patients (10.2 vs 4.7 kPa; P=0.0001), and considering patients with TE values >12 KPa, the 80% (8/10) were RNA positive. To investigate the role of iron overload and HCV infection in developing liver damage, all patients were divided in 6 groups based on HCV RNA positivity/negativity and serum ferritin levels (<1000 ng/mL, 1000-2500 ng/mL, >2500 ng/mL). In patients with serum ferritin levels <2500 ng/mL HCV RNA positive group showed significant higher levels of ALT compared to HCV RNA negative (serum ferritin levels <1000 ng/mL P=0.007; serum ferritin levels 1000-2500 ng/mL P=0.005); while HCV RNA positive patients with serum ferritin levels >2500 ng/mL had increased ALT levels but not statistically significant compared to HCV RNA negative. TE values in HCV RNA positive patients were respectively 7 kPa in patients with ferritin<1000 ng/mL and 9.6 kPa in patients with ferritin between 1000 and 2500 ng/mL. So far 25 (35,3%) RNA positive patients underwent antiviral treatment and a sustained viral response was observed only in 8/25 (31%) patients. Among RNA positive we observed 4/71 (5.6%) cases of hepatocellular carcinoma (HCC) in patient aged 45±5 years, a number significantly higher than HCV RNA positive non Thalassemia population of similar age; no one of them underwent antiviral treatment. *Conclusions.* Although chronic active infection plays a main role in developing liver fibrosis, the highest stage of fibrosis were observed in HCV RNA positive patients and with severe iron overload. These data confirm that HCV infection and iron overload are synergistic risk factors in developing fibrosis and cirrhosis that may lead to HCC. Thus it's mandatory to consider antiviral treatment in HCV positive TM patients.

0502

MOLECULAR ANALYSIS IN FAMILIAL CONGENITAL ERYTHROCYTOSIS: NOVEL MUTATIONS IN EPOR AND VHL GENES

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Background. Congenital erythrocytosis results from 1) inherited intrinsic defects in red blood cell precursors that cause increased responsiveness to EPO due to a mutation in EPO receptor gene (EPOR); 2) inherited defects in hypoxia sensing pathway due to mutation in the Von Hippel-Lindau gene (VHL), in the prolyl hydroxylase domain 2 gene (PHD2) or in the hypoxia-inducible factors gene (HIF); 3) high affinity hemoglobin variants; 4) 2,3-difosfogliceratomutase (DPGM) enzyme deficiency. The inheritance is autosomic: dominant for high-affinity Hb and EPOR mutations and recessive for DPGM, VHL and PHD2 gene mutations. Conversely, in a great number of congenital erythrocytosis the underlying defect remains unknown. *Aims.* To characterise the molecular basis of the erythrocytosis in a group of patients studied in our laboratory. *Methodology.* We studied 61 subjects, from 40 different families, with erythrocytosis. Patients were either regularly followed in our Haematology clinic or referred from other Centres. Non haematological causes associated with erythrocytosis were excluded. Laboratory testing was guided by the clinical history and EPO levels and included: exclusion of JAK2 V617F mutation by ASO-PCR, Hb analysis by high-performance liquid chromatography, DPGM activity quantification and HBB, HBA, VHL, EPOR (exons 7- 8), DPGM and PHD2 genes sequencing. *Results.* In this study we identified: in 15 subjects (11 families) eight different high-affinity Hb variants; in 10 subjects (4 families) three different mutations in the exon 8 EPOR gene; in 1 patient a homozygous missense mutation in the exon 3 VHL gene. One of the EPOR mutations, a mutation in CD487 (AAC-AGC) was already reported, the other two are novel mutations in exon 8: a nonsense 2 base pair deletion (-TC) starting in nucleotide 5997, resulting in a premature stop codon and a misense CD 437 (CGT-CAT) mutation. In the VHL gene a novel misense mutation, CD196 (AAA-GAA) in homozygote state was detected in a Spanish woman, with consanguineous parents. *Conclusions.* We were able to identify the congenital erythrocytosis molecular aetiology in 16/40 unrelated families (26/61 subjects). The presence of a high-affinity Hb variant was the commonest cause (11 families). Two novel mutations in EPOR and one in VHL, were identified. None of the subjects showed mutations in DPGM or PHD2 genes. In the remaining 24 families the erythrocytosis genetic underlying defect(s) remain elusive, needing further investigations.

0503

LOW-DOSE RITUXIMAB IN IDIOPATHIC AUTOIMMUNE HAEMOLYTIC ANAEMIA

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Background. Conventional therapy of warm autoimmune haemolytic anaemia (WAIHA) include administration of corticosteroids and immunosuppressive agents, or splenectomy, whereas no effective treatment exists for cold hemagglutinin disease (CHD). A substantial proportion of patients with WAIHA do not respond to or relapse after corticosteroid therapy and may experience clinically relevant side effects. Favorable responses to rituximab at standard doses (375 mg/m^2 weekly for 4-6 courses) have been reported in both WAIHA and CHD, idiopathic or secondary, as well as in other autoimmune diseases, such as rheumatoid arthritis and primary immune thrombocytopenia. Recently, low dose (LD)-rituximab (100 mg fixed dose weekly for 4 courses) has been proven effective in patients with autoimmune cytopenias, particularly immune thrombocytopenia. *Aims.* To evaluate the safety, activity and the duration of the response of LD-rituximab associated with standard oral prednisone (PDN) as first line therapy in newly diagnosed WAIHA and CHD, and as second line therapy in WAIHA relapsed after standard oral PDN. *Methods.* In this single-arm prospective pilot study, LD-rituximab was administered at 100 mg fixed dose weekly on days +7, +14, +21, +28 along with standard oral PDN (1 mg/kg/die p.o. from day +1 to + 30, followed by quick tapering: 10 mg/week until 0.5/mg/kg/die, then 5 mg/week until stop). Complete and partial initial responses (iCR and iPR) were defined as Hb > 12 g/dL and > 10 g/dL at month +2 from the beginning of therapy, respectively; sustained response (SR) was defined as Hb > 10 g/dL at month +6 and +12, in the absence of any treatment. *Results.* Seventeen patients (10 female, 6 male; median age 51 yrs, range 28-72) were enrolled after informed consent. An iCR and iPR were observed in 10 (59%) and 4 (24%) out of 17 patients, respectively; the median Hb level increased from 9.2 g/dL (range 4.4-12.3) at enrolment to 12.4 g/dL (range 9.1-15.3) at month +2. A SR at month +6 was observed in 12 out of 12 evaluable patients, and in the first 6 patients enrolled at month +12. One patient relapsed at month +16 and was retreated with the same protocol with iPR at month +18, and another patient at month +7, again treated with iPR at month +9. No side effects or serious adverse events were observed. For 9 relapsed AIHA patients laboratory data (Hb, LDH, reticulocytes) and steroid administration were available before LD-rituximab treatment: a lower cumulative dose (roughly 50%) of steroid was administered to patients during LD-rituximab study compared with previous therapy, without significant differences in the general trend of Hb, LDH, and reticulocytes. These preliminary results seem to indicate that the addition of LD-rituximab to standard corticosteroid therapy is a feasible and active treatment in AIHA. Data on SR are intriguing, particularly regarding the possible steroid sparing effect of LD-rituximab, but need to be confirmed in larger survey and after longer follow up.

0504

BLOOD TRANSFUSION USAGE AMONG PATIENTS WITH SICKLE CELL DISEASE - A SINGLE INSTITUTION EXPERIENCE OVER TEN YEARS

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Background. A major option in the treatment of sickle cell disease (SCD) is the transfusion of red blood cells on a chronic or intermittent basis. Long term or life-long transfusion for primary or secondary prevention of stroke is recommended (Adams *et al.*, 1998, Adams and Brambilla, 2005, Lusher *et al.*, 1976) but in recent years there has been expanding usage of blood transfusion with no clear evidence base. *Aims.* To examine the trend in blood usage among adult patients with SCD in a single institution. *Method.* The study includes adult patients (over 16 years of age) attending King's college Hospital (KCH), London, over a ten year period between 1st of January 2000 to 31st of December 2009. A total of 659 patients were identified from matching the KCH SCD database with in- and outpatients attendance. We then used this list to interrogate PathNet and WinPath (transfusion databases) and KCH electronic patient record (EPR) system for details of blood issued including indications for transfusion, number of units and method of transfusion.

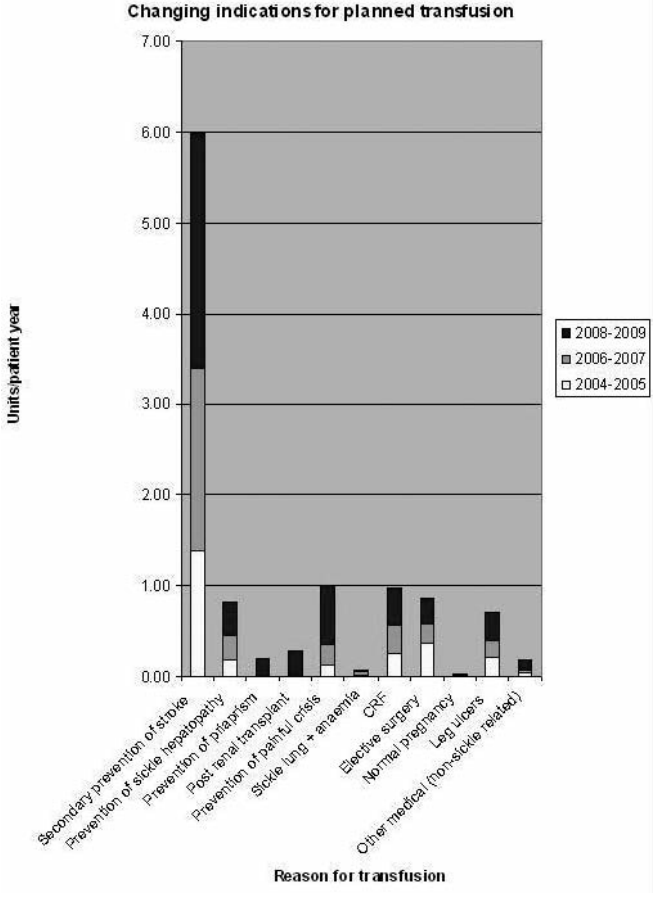

Figure 1. Changing indications for planned transfusions.

Results. The KCH cohort of SCD increased from 313 patients per year to 435 patients per year from 2000 to 2009 but the proportion of genotypes has remained stable during this time (mean%= HbSS 64%, HbSC 28%, SB+ 5%, SBo 3%). During this time period 44% of the patients were transfused at least one unit of blood. Although HbSS patients comprised 64% of the cohort they used 84% of all blood transfused. Further, 93% of all blood transfused was given to only 9% of patients thus defining this group as having a relatively more severe phenotype. The percentage of patients transfused each year has increased from 16% in 2000 to 19% in 2009, and mean units per patient increased significantly (P<0.005) from 12 (95% CI 7.6 to 16.2) to 21 (95% CI 15.7 to 25.9). Increase in blood use is most marked between 2005 and 2009 and is due to increased exchange transfusion, the majority for secondary prevention of stroke. Indications for planned transfusion have also expanded in our cohort (Figure 1). *Summary.* Blood use in the Kings sickle cell disease population has increased in the past ten years due to increase in exchange transfusion and expanding indications such as preventive therapy for sickle hepatopathy, priapism and control of acute pain (failed hydroxycarbamide therapy). The majority of blood is given to a small proportion of patients with severe disease; further evidence of the remarkable variation in disease severity.

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0505

HIGH PREVALENCE AND MORTALITY ASSOCIATED WITH THROMBOEMBOLISM IN ASIAN PATIENTS WITH PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH)

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Introduction. PNH is a hematopoietic stem cell disorder in which uncontrolled activation of the terminal complement system leads to significant ischemic morbidities with shortened lifespan. Life-threatening thromboembolism (TE) is the most feared complication of PNH, accounting for up to 40-67% of patient deaths. It is estimated that 40% of PNH patients experience a clinically evident TE and 60% of patients without clinically diagnosed TE demonstrate TE by high-sensitivity MRI, indicating the ongoing thrombotic risk in most patients with PNH. *Aims.* To understand the impact of TE in Asian patients with PNH, we retrospectively analyzed medical charts of 286 PNH patients from national data registry in South Korea over the last 41 years.

Table.

Predictor of Mortality	Odds ratio; P value*
TE	Odds ratio 7.0; P<0.0001
Abdominal Pain	Odds ratio 2.2;P=0.046
Predictor of TE	**Odds ratio; P value***
Abdominal Pain	Odds ratio 3.6; P=0.0004;
Chest Pain	Odds ratio 2.6; P=0.024
Dyspnea	Odds ratio 2.4; P=0.015
Hemoglobinuria	Odds ratio 2.1; P=0.046
Sites of Thromboembolism Events	**% of Total TE**
Venous TE	**60 %**
Deep vein thrombosis	18%
Mesenteric/visceral vein	12%
Pulmonary embolism	10%
Hepatic portal vein	8%
Renal vein thrombosis	6%
Dermal	1.5%
Gangrene	1.5%
Cerebral venous occlusion	1.5%
RUL infarction	1.5%
Arterial TE	**39%**
Cerebral arterial occlusion	15.0%
Coronary arterial occlusion	13.0%
Myocardial infarction	(7.5%)
Unstable angina	(4.5%)
Mesenteric/visceral artery	9.5%
Retinal artery obstruction	1.5%

*Fisher exact test was used

Results. Patient ages ranged from 8 to 88 years (median 37 years), median PNH duration was 7.8 years (1 month to 41 years), and median PNH granulocyte clone size was 49%. History of another bone marrow disorder (BMD was observed in 45% of patients. TE was a strong predictor of mortality (P<0.0001; odds ratio 6.99; 95% CI 3.2 - 15.2) and accounted for 46% of patient deaths. TE was reported in 15% (43/286) of patients, of which 40% (17/43) had > 2 recorded TEs. Median age at first TE was 40 years. The likelihood of a TE was unrelated to the presence of another BMD; 40% of TE patients had BMD vs 60% of non-TE patients had BMD. TE likelihood was not influenced by granulocyte clone size: median clone size was 52% in patients with TE, 20% of patients with TE had clone size <30%, and clone size was 50% in patients without TE. TE manifested at both venous (60%) and arterial (39%) sites. Specific venous sites include DVT (18%), mesenteric/visceral vein (12%), PE (10%), hepatic portal vein (8%), and renal vein thrombosis (6%). Arterial sites were particularly common: cerebral arterial occlusion (15%), coronary artery (13%), and mesenteric/visceral artery (9.5%). The presence of abdominal pain is a significant predictor of TE (Odds ratio 3.6, 95% CI 1.8-7.4, P=0.0004) and mortality (Odds ratio 2.2, 95% CI 1.0 - 4.5, P=0.046). Additionally, dyspnea (Odds ratio 2.4, 95% CI 1.2 - 4.6, P=0.015), hemoglobinuria (Odds ratio 2.1, 95% CI 1.0 - 4.2, P=0.046), and chest pain (Odds ratio 2.6, 95% CI 1.2 - 6.0, P=0.024) were also significant predictors for TE. TE is an important signal to test for PNH. TE was reported in 21% (9/43) of patients before the diagnosis of PNH and in 21% (9/43) of TE patients within 1 month of diagnosis. *Conclusions.* These data indicate that the prevalence and impact of TE in Asian patients with PNH is similar to that in other populations. TE occurs in young patients, in venous sites and is particularly prominent in arterial locations. TE risk appears to be strong across the spectrum of granulocyte clone sizes and the presence of concomitant BMD does not diminish the risk for TE. Abdominal pain is strongly associated with TE in Asian patients. Most importantly, TE is a significant contributor to early mortality in Asian PNH patients.

0506

CLINICAL SYMPTOMS OF HEMOLYSIS ARE PREDICTIVE OF DISEASE BURDEN AND MORTALITY IN ASIAN PATIENTS WITH PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH)

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Background. PNH is a progressive and life-threatening disease characterized by chronic complement-mediated hemolysis. The progressive morbidities and mortality associated with PNH, including life-threatening thromboembolism (TE), renal dysfunction, pulmonary hypertension, disabling fatigue, abdominal pain and dysphagia, lead to diminished quality of life. Failure to recognize the clinical consequences of chronic hemolysis results in delayed diagnosis and, more importantly, in progressive morbidities and disease burden. The frequency and type of clinical symptoms, and the relation of these symptoms to severe morbidities and early mortality has not been previously examined in Asian PNH patients. *Aims/Methods.* To describe the clinical manifestations and disease burden of Asian PNH patients, we retrospectively analyzed medical records 286 PNH patients from national data registry in South Korea. *Results.* The median patient age was 37 years (range: 8 to 88 years) and PNH duration was 7.8 years. At diagnosis median PNH granulocyte clone was 49% and LDH was 3.9-fold above upper limit of normal (ULN). Forty-five percent (45%) of patients had history of concomitant bone marrow disorder. Median platelet count was 99×10^9/L and median ANC was 1.2×10^9/L, 21% with ANC $<1.0\times10^9$/L (with mean LDH 3.8 fold above ULN). Corticosteroids were employed in 78% and immunosuppressive treatments (ATG or CsA) in 20% of patients. Fatigue was common and reported in 76% of patients. TE was detected in 15% of patients, 21% before the diagnosis of PNH. TE was a strong predictor of mortality (P<0.0001; odds ratio 6.99; 95% CI 3.2-15.2), yet only 14% of patients were treated with antithrombotics. Late-stage kidney disease (medical diagnosis of renal impairment or documented GFR <60 mL/min/1.73 m^2) was somewhat more common than observed in Western studies and observed in 27% (36/133) of evaluable Asian patients following diagnosis. Impaired hepatic function was reported in 6% of patients, 47% after the diagnosis of PNH diagnosis. Pain was reported by 58% of patients. Medical intervention for pain was required in 47% of these patients: NSAIDs (38%), opioids (18%). Abdominal pain was reported in 46% of patients and treatment accounted for 75% of the total opioid use. Abdominal pain was a strong marker for clinical TE: 72% of patients with TE had evidence of abdominal pain. Abdominal pain significantly predicted TE (Odds ratio 3.6, 95% CI 1.8-7.4, P=0.0004) and mortality (Odds ratio 2.2, 95% CI 1.0-4.5, P=0.046). Clinical symptoms of PHT were prominent (41% of patients): shortness of breath (36%) and chest pain (12%). Furthermore, chest pain also was a significant predictor of TE (Odds ratio 2.7, 95% CI 1.2-.2, P=0.024); 29% of patients with chest pain had a TE. *Conclusions.* These data demonstrate that Asian PNH patients frequently suffer disabling symptoms and progressive disease burden including late stage kidney disease, liver dysfunction, and symptoms of PHT. Moreover, prominent symptoms such as abdominal pain and chest pain are predictors of TE. Finally, TE is a strong predictor of mortality in Asian patients. Despite medical intervention with supportive care, including 78% corticosteroid use, patients continued to demonstrate disabling symptoms, progressive complications and early mortality.

Table.

Major Symptoms of PNH	Percent of Patients
Thromboembolism	15%
Late-stage kidney disease (after diagnosis)	27%
Impaired hepatic function	6%
Pain	58%
Abdominal pain	46%
Hemoglobinuria	58%
Symptoms of pulmonary hypertension (PHT)	
Shortness of breath	36%
Chest pain	12%
Medical Treatments	
Non-specific Immunosuppressive treatment	
Corticosteroids	78%
Other immunosuppressants (CsA or ATG)	20%
Treatment or prevention of TE	
Antithrombotic medication (warfarin, heparin)	14%
Treatment of pain	
Anti-inflammatory medication	38%
Opioid medication	18%
Treatment of abdominal pain	
NSAID or opioid medications	40%

0506a

ERYTHROCYTOSIS ASSOCIATED WITH A NOVEL MISSENSE MUTATION IN THE HIF2A GENE

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Background. Erythropoietin (EPO) is the central regulator of red blood cell mass. The EPO gene is regulated by the key transcription factor Hypoxia Inducible Factor (HIF) which is composed of an oxygen-sensitive α subunit (HIF-1α, HIF-2α, or HIF-3α) and an oxygen-insensitive β subunit. In this pathway, Prolyl Hydroxylase Domain protein 2 (PHD2) hydroxylates two prolyl residues in HIF-α, which in turn promotes HIF-α degradation by the von Hippel Lindau tumor suppressor (VHL) protein. Erythrocytosis is an uncommon disorder characterized by abnormally elevated red cell mass. Some cases can be attributed to mutations in proteins of the oxygen-sensing pathway that regulates HIF such as VHL and PHD2. Recently, studies have shown that mutations in the HIF2A gene comprise a third cause of erythrocytosis attributable to defects in the oxygen-sensing pathway. These studies implicate HIF-2α as the central HIF-α isoform regulating EPO in human adults. All reported HIF2A gene mutations to date affect residues that are C-terminal to the primary hydroxylation site in HIF-2α, Pro531. They variably affect the interaction with PHD2 and/or VHL. These mutations, while affecting different proteins in the oxygen-sensing pathway, all lead to aberrant upregulation of HIF-2α. *Aims.*We describe a patient with a novel HIF2A mutation associated with erythrocytosis. This mutation is predicted to result in a seemingly conservative p.Asp539Glu amino acid substitution. Notably, this is perhaps the most subtle mutation thus far described. *Methods.* The patient is a 17-year old girl of North African ancestry with elevated hemoglobin levels and red blood cell counts, respectively 17.7 g/dL and 6.3×10^{12}/L. The inappropriately high level of EPO (25 IU/L) together with the absence of any pulmonary, cardial, or renal abnormalities suggested a genetic cause of secondary erythrocytosis. Therefore, the genes encoding hemoglobin, bisphosphoglyceratemutase, PHD2, VHL, and HIF-2α were investigated by DNA sequence analysis. *In vitro* PHD2 binding assays were performed employing in vitro translated GAL4-HIF-2α fusion proteins and recombinant PHD2. VHL binding assays were performed by assessing the capacity of hydroxyprolated HIF-2α peptide to compete with hydroxyprolated HIF-1α binding for in vitro translated VHL. Hydroxyproline-531-substituted wild type or Asp539Glu HIF-2α peptides as well as N-terminal biotinylated Hyp-564 HIF-1α peptide were commercially obtained. *Results.* DNA sequence analysis showed no abnormalities other than a novel heterozygous missense mutation in HIF2A, predicting a Asp539Glu substitution at residue 539. *In vitro* PHD2 binding assays showed that wild type HIF-2α binds with easily detectable affinity to PHD2 whereas the p.Asp539Glu mutation weakens this interaction. In addition, VHL binding assays showed that VHL binds with high affinity to Hyp-HIF-1α with wild type Hyp-HIF-2α acting as an effective competitor. The p.Asp539Glu mutation diminishes the capacity of the Hyp-HIF-2α peptide to compete for VHL binding. *Summary/conclusions*. We conclude that the p.Asp539Glu mutation impairs the interaction of HIF-2α with both PHD2 and VHL. This is similar to most HIF-2α mutations studied to date. These findings support the notion that HIF-2α is the critical isoform regulating EPO in human adults and indicate that a seemingly conservative substitution is sufficient to disrupt this pathway.

Stem cell transplantation 1

0507

SUPERIOR GRAFT-VERSUS-LEUKEMIA EFFECT ASSOCIATED WITH TRANSPLANTATION OF HLA-MISMATCHED/HAPLO-IDENTICAL COMPARED WITH HLA-IDENTICAL SIBLING DONOR GRAFTS FOR PATIENTS WITH HIGH RISK ACUTE LEUKEMIA

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Background Great progress has been made in haploidentical donor (HID) hematopoietic stem cell transplantation (HSCT). over the past 20 years, and it has become a feasible option for leukemia patients without a HLA-identical sibling donor (ISD), especially with high-risk features. It has been speculated that HID HSCT may potentially exert a strong GVL effect, but there have been no comparative clinical studies to confirm this hypothesis. *Aims.* The purpose of this non-randomized, single-center study was to comparatively analyze transplantation outcomes in a consecutive series of high-risk acute leukemia patients who underwent HSCT from either HID or ISD without *in vitro* T cell depletion (TCD) at our institute. *Methods.* The study was approved by the Institutional Review Board of the Peking University Institute of Hematology. All included patients were informed and signed an informed consent form. Consecutive patients with high-risk acute leukemia (patients in CR3 or beyond, non-remission, or CR1 with high-risk cytogenetics, such as t (4; 11) or t (9; 22), n=117) receiving HSCT from either an ISD (n=36) or a HID (n=81) between January 2005 and April 2009 were enrolled. If a matched sibling donor was unavailable as a first treatment option, patients without a suitable closely HLA-matched unrelated donor, or whose disease status left insufficient time for an unrelated donor search, were eligible for HID HSCT. The conditioning therapy for the HID group consisted of a modified BUCY2 plus ATG (thymoglobulin), while patients in the ISD group received modified BUCY2 without ATG.

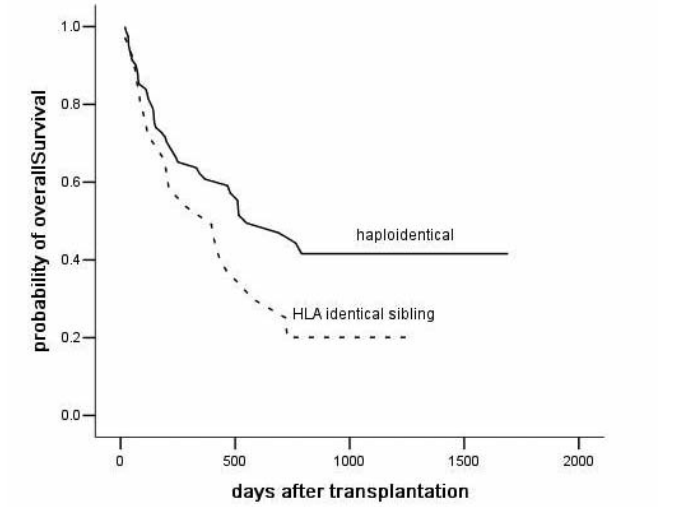

Figure. Probability of OS after ISD or HID HSCT

Results. Full engraftment was achieved in 98% patients in the HID group and 97% in the ISD group. Patients engrafted to absolute neutrophil counts exceeding 0.5×10^9/L at a median time of 13 days in the HID group and 16 days in the ISD group (P=.061). The cumulative incidences of grades II-IV acute GVHD in the HID and ISD cohorts were 49% and 24%, respectively (P=.014), with a relative risk (RR) of 2.99 (95% CI, 1.25-7.21) (P=.014). The 2-year cumulative incidences of chronic GVHD in the HID and ISD cohorts were 62% and 39%, respectively (P=.11), with a relative risk (RR) of 1.52 (0.69-3.34) (P=.30). The 2-year cumulative incidence of relapse was significantly lower in HID (26%) than in ISD patients (49%) (P = .008) with RR = 0.21 (0.08-0.53) (P=.001). The 2-year cumulative incidence of non-relapse mortality was comparable in recipients of HID (34%) and ISD grafts (38%) (P=.85).

The 3-year probability of overall survival was higher in HID patients (42%) than in ISD (20%) (P=.048) patients. The 3-year LFS in the HID group was 42% versus 15% in the ISD group (P =.029). *Summary/Conclusions* The current study showed that lower relapse rate, similar engraftment rate, and higher survival probability was achieved with HID patients than with ISD patients. The results suggest that HID HSCT might achieve a better anti-leukemia effect for high-risk acute leukemia patients.

0508

MONITORING OF WT1 EXPRESSION IN PB AND CD34+ DONOR CHIMERISM OF BM PREDICT EARLY RELAPSE IN AML AND MDS PATIENTS AFTER HEMATOPOIETIC CELL TRANSPLANTATION WITH REDUCED INTENSITY CONDITIONING

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*Background.*HCT following reduced intensity conditioning (RIC) relies mainly on immunological effects for disease control. The early detection and quantification of minimal residual disease and the timely adjustment of immune suppression are therefore particularly important in this setting. *Aims.* Since appropriate disease-specific gene markers are not always available in patients with acute myeloid leukemia (AML) or myelodysblastic syndrome (MDS), the potential of both donor chimerism and Wilms Tumor gene 1 (WT1) expression to provide quantification of MRD was investigated. *Patients and Methods.* Eighty eight consecutive patients with AML (n=68) and intermediate/high-risk MDS (n=20) were analyzed. Patients were 61 (median; range 22-74) years old and in CR1 (n=41), CR2 (n=25), or more advanced disease status (n=22). Grafts were obtained either from related (n=22) or unrelated (n=66) donors. Conditioning regimens consisted of fludarabine 30 mg/kg BW day -4 to -2 (n=84) and total body irradiation with 2 Gy at day 0 (n=88), and post-transplant immunosuppression employed cyclosporin A and mycophenolate mofetil. Total donor chimerism (TDC, n=86 patients, 221 samples), CD34+-chimerism (n=86, 196 samples) and disease-specific molecular markers detected by FISH (DSM, n=38, 70 samples) were all determined prospectively from bone marrow (BM) samples at baseline and on days +28, +56 and +84 post-transplant. WT1 expression was analyzed retrospectively by RT-PCR from stored peripheral blood (PB) samples (n=86, 260) from the same time points. *Results.* With a median follow-up of 12.3 (range 1.3-64.9) months, 32 (37.2%) patients relapsed (defined by BM blasts >5%). Since complete results from all techniques were available up to day 84, we analyzed the diagnostic power of all methods to predict hematological relapse one month in advance up until the fourth month after HCT (n=21 patients, 24%, 66% of all relapses). First, we estimated the value of the three different prospective MRD techniques (DSM, TDC and CD34+ chimerism) using Receiver Operating Curves (ROC). Relapse was predicted 1 month in advance by a decrease of $CD34^+$-chimerism of ≥ 5% [P= 0.001, area under the curve (AUC) = 0.895], but not by TDC or DSM. The cut-off value of 5% decrease in $CD34^+$ chimerism in a one month period achieved a sensitivity of 71% and specificity of 91%. In comparison, WT1 expression was similarly associated with a pending relapse (P< 0.0001, AUC = 0.855). The optimal cut-off value of 24 WT-1 copies per 10000 ABL copies was assessed by ROC and resulted in a sensitivity of 79% and specificity of 89%. In a logistic regression model, we estimated the ratios of the odds of relapsing within the next month. WT1 achieved a higher odds ratio (27.6/1) than $CD34^+$ chimerism (23.9/1). Combining both techniques yielded a specificity of 98% and an odds ratio of 61/1. *Conclusions.* WT1 expression in PB and $CD34^+$ chimerism in BM are superior to full donor chimerism and disease-specific markers determined by FISH in predicting relapse

0509

STABLE VESSEL GENERATION TROUGH APPLICATION OF BLOOD-DERIVED ENDOTHELIAL COLONY FORMING PROGENITOR CELLS (ECFCS) AND MESENCHYMAL STROMAL / STEM CELLS (MSCS)

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ECFCs have recently been described as vascular progenitor cells with robust proliferative potential and vessel-forming capacity. This study was performed to establish conditions for the generation of stable vessels by ECFC/MSC-cotransplantation. MSCs were propagated as previously described. ECFCs were isolated with a novel recovery strategy and propagated under animal protein-free culture conditions with pooled human platelet lysate (pHPL) replacing fetal bovine serum (FBS). ECFC long-term proliferation potential was monitored and phenotype was analyzed by flow-cytometry and immune- cytochemistry. Functionality was studied during vascular network assembly *in vitro* and in human vessel formation in immune-deficient mice *in vivo*. Genomic stability was assayed with chromosome G-banding and array-comparative genomic hybridization (array-CGH). Additionally we compared telomere-length and telomerase-activity of ECFCs at different time points of culture with flow-fluorescence *in situ* hybridization (Flow-FISH) and telomere repeat amplification protocol-assay (TRAP). A mean of four ECFC colonies/mL of peripheral blood could be recovered. The progeny of these cultures could be expanded to mean $1.5\pm0.5\times10^8$ ECFCs within 11-25 days. ECFC purity, immune phenotype and sustained proliferation potential for >30 population doublings with preserved progenitor hierarchy could be confirmed following analysis. Karyotyping and array-CGH revealed genomic stability. Large-scale expanded ECFCs functioned even after cryopreservation to form complex vascular networks *in vitro* and assembled stable $CD31^+$/$Vimentin^+$/von Willebrand factor+ human vessels when transplanted with MSCs *in vivo*. Direct connection to murine circulation of the developed human vessels was indicated by a rich content of $Ter119^+$ murine erythrocytes. This demonstrates that ECFC/MSC-cotransplantation results in generation of functional and stable human vessels. The procedure should help to set a new standard for the study of therapeutic applicability and risk profile of vessel-forming ECFC-based investigational new drugs.

0510

A NEW PRE-TRANSPLANT PREDICTIVE MODEL FOR EVENT FREE SURVIVAL INCORPORATING FUNCTIONAL IMAGING USING FDG-PET IN LYMPHOMA PATIENTS UNDERGOING HDC & ASCT

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Background. There are emerging data indicating poor outcome in diffuse large cell lymphoma (DLCL) and Hodgkin's Lymphoma (HL) patients with positive 18F-fluorodeoxyglucose positron emission tomography ((FDG-PET) before HDC ASCT. Large studies to evaluate various prognostic factors along with FDG-PET in this setting are very limited. *Aims.* This study evaluates the impact of PDG-PET among other prognostic / predictive factors. We also constructed a predictive model based on pre transplant FDG-PET results and other factors for post HDC ASCT residual / progressive disease and relapse in patients with DLCL and HL undergoing HDC ASCT. *Methods.* From 2003 to September 2009, 215 consecutive patients with HL and DLCL underwent HDC ASCT. Of these, 127 patients had FDG-PET after salvage chemotherapy / prior to HDC ASCT. ESHAP was used as salvage chemotherapy, responding DLCL or responding / stable HL patients had BEAM as HDC. Patient had CT scan and FDG-PET before starting ESHAP, after 2-3 cycles of ESHAP / prior to HDC ASCT and 100 days post ASCT. FDG-PET "positive study" was defined as study showing evidence of disease and "negative study" as no evidence of disease. Disease specific (DS) event is defined as presence of persistent disease, progression or relapsed disease after ASCT. DS event free survival (DS-EFS) was calculated from day 0. Logistic regression (univariate analysis) was used to identify the significance of various factors at relapse / progression on DS-EFS (gender, histology, prior XRT, age at ASCT 30 vs > 30, LDH, PS 0,1 vs higher, B symptoms, spleen involvement, CT positive vs negative, extranodal involvement, stage I-II vs III-IV, bulky disease, mediastinal involvement, refractory vs relapsed, FDG-PET positive vs negative. Factors with P=< 0.1 were selected for multivariate analysis. Each

patient was scored with 0, 1, 2 or 3 depending upon the number of significant risk factors identified. DS-EFS was calculated using Kaplan-Meier Method and comparison between groups using log-rank test. *Results.* There were 73 (58%) male and 54 (42%) female: DLCL: 34 (27%) and HL: 93 (73%). Relapsed: 59 (46%), refractory 68 (54%). Median age at ASCT was 28 years (14 to 63). Median follow-up of all patients from ASCT is 18 months (1.2 to 83 months); alive patients 21 months. Mmultivariate analysis showed FDG-PET positive (P=0.0001), mediastinal involvement (P=0.008) and refractory disease (P=0.04) as significant factors. As of February 28, 2010, of these 127 patients, 45 (35%) had a DS-EFS event, DS-EFS is 65%. DS-EFS for PET negative vs. positive patient is 72% vs. 40% at 36 months (P=0.0001). DS-EFS for patients with score 0 (100%), score 1 (66%), score 2 (51%) and score 3 (26%) showing a very significant impact (P=<0.0000) ((survival graph). *Conclusions.* Prior to HDC ASCT, FDG - PET scan result is the most important predictor of DS-EFS among mediastinal involvement and refractory disease. Based on these factors, a score can be used to predict treatment failure in these patients. Higher score indicates increasing risk of residual disease / progression or relapse. These patients are potential candidate for more aggressive and experimental therapies.

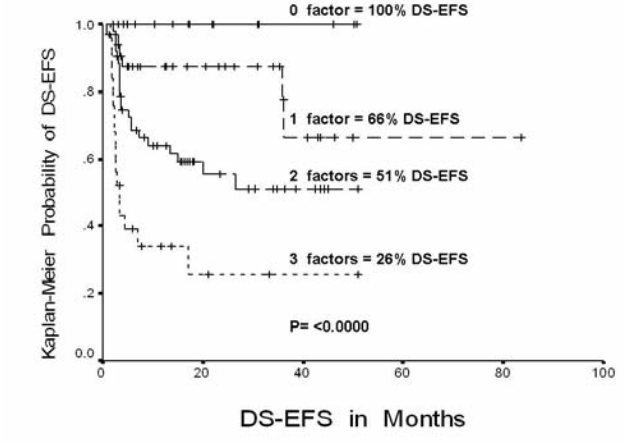

Figure. Prognostic score and KM Probability of DS-EFS

0511

INDUCTION OF GVHD BY HEMATOPOIESIS-SPECIFIC T CELLS MAY OCCUR DURING AN ONGOING PROFOUND GVL REACTION DUE TO ICAM-1 MEDIATED INDUCTION OF COLLATERAL DAMAGE

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Graft versus host disease (GvHD) after allogeneic stem cell transplantation (SCT) may occur when donor T cells respond to antigens specifically expressed on cells of the recipient, such as minor histocompatibility antigens (mHags). It has been hypothesized that ubiquitously expressed mHags, like HY and ADIR-1F may be targets for both GvHD and graft versus leukemia (GvL), whereas hematopoiesis-restricted mHags such as HA-1 may give rise to a more specific GvL effect without GvHD. However, it has been reported that mismatching for HA-1 is correlated with the development of GvHD. In addition, there is clinical evidence that the presence of a high tumor load leading to a profound GvL reaction may be associated with the induction of GvHD. Based on these observations, we hypothesized that hematopoiesis-restricted T cells may have the capacity to induce collateral damage to surrounding non-hematopoietic tissues during an ongoing profound immune response against hematopoietic cells of the patient. To test this hypothesis we investigated in-vitro whether mHag-specific cytotoxic T lymphocytes (CTLs), in the presence of mHag positive hematopoietic targets, could lead to lysis of surrounding mHag-negative primary human fibroblasts in a 4, 8 and 20hrs chromium release assay. No damage to fibroblasts occurred under steady state conditions. However, in a pro-inflammatory environment mimicked by IFNg pretreatment of fibroblasts, HA-1-specific CTLs optimally activated by HA-1 positive EBV-LCL (S/R ratio 10/1), exerted 40% (25-60%) bystander cytotoxicity to the surrounding fibroblasts at a 10/1 T cell/fibroblast-ratio after 20 hrs. T cell activation was required for the induction of this collateral damage since no bystander kill was observed when HA-1-specific CTLs were exposed to HA-1 negative EBV-LCL. Using a transwell system we demonstrated that this bystander kill was not mediated by soluble factors like cytokines, since direct T cell-fibroblast interaction was required. To investigate how mHag-specific CTLs could interact with the mHag negative fibroblasts we used imaging techniques using fluorescent microscopy. As expected no strong association of mHag-specific CTLs with mHag negative fibroblasts was observed under steady state conditions. However, under pro-inflammatory conditions a firm attachment of mHag-specific CTLs to fibroblasts was seen in the absence of specific peptide/MHC recognition. Since ICAM-1 was significantly upregulated on the fibroblasts after IFNg pre-treatment, we analyzed the requirement of ICAM-1 expression by transduction of ICAM-1 into fibroblasts. This enforced expression of ICAM-1 appeared to be sufficient to achieve the firm T cell-fibroblast interactions and allowed the mHag-specific T cells to induce collateral damage even without IFNg pretreatment. In conclusion, these data indicate that hematopoiesis-specific T cells, activated during a profound GvL immune response may create a pro-inflammatory environment, which allows them to attach to surrounding non-hematopoietic tissues in the absence of expression of relevant peptide/MHC complexes, resulting in the induction of collateral damage.

0512

PLASMA BRAIN NATRIURETIC PEPTIDE PREDICTS HEPATIC VENO-OCCLUSIVE DISEASE AND MORTALITY IN ALLOGENEIC STEM CELL TRANSPLANTATION

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Background. Hepatic veno-occlusive disease (VOD) is a life-threatening complication after hematopoietic stem cell transplantation (HSCT) and featured with unexplained fluid retention, jaundice, and right hypochondrial pain with hepatomegaly. Because brain natriuretic peptide (BNP) is a 32-amino-acid neurohormone synthesized in ventricular myocardium and secreted in response to volume expansion, we hypothesized that plasma BNP concentrations indicate onset and/or severity of VOD and serve as a prognostic marker after HSCT. *Methods.* We retrospectively analyzed the association of plasma BNP with the onset of VOD and early mortality after allogeneic HSCT in 33 consecutive adult patients who underwent allogeneic HSCT at our institution between February 2008 and February 2009. Plasma BNP concentrations were measured once before transplantation for routine workup and weekly after transplantation, using fluorescence enzyme immunoassay. The diagnosis of VOD was based on modified Seattle criteria and the severity of VOD was defined according to the established criteria. *Results.* A total of 12 (36.3%) patients developed VOD (mild to moderate in 6 and severe in 6) between 1 and 15 days (median, 9.5 days) after transplantation. Plasma BNP concentrations were similar before and on day 0 between patients with and without VOD, but significantly increased on day 7 and later in those with VOD. Peak plasma BNP concentrations before engraftment in patients with VOD were significantly elevated compared with those without (median values, 1686.9 pg/mL vs. 87.6 pg/mL; P=0.01). Among patients with VOD, peak BNP was associated with its severity (3678.0 pg/mL in severe VOD vs. 525.1 pg/mL in mild to moderate VOD; P=0.02). We investigated the impact of plasma BNP on early mortality using peak plasma BNP concentrations before day 14 because most patients presented VOD before day 14. ROC curve analysis showed a BNP cutoff value of ≥380 pg/mL could effectively differentiate nonsurvivors from survivors at day 100, with a sensitivity of 85.7% and a specificity of 84.6%. We investigated the associations between plasma BNP concentrations and these conditions because plasma BNP concentrations can be increased in various diseases including heart failure and sepsis. However, there was no significant correlation between peak BNP concentration before engraftment and documented infection (P=0.72). With respect to cardiac function, pretransplant left ventricular ejection fraction (LVEF) showed no correlation with peak plasma BNP concentrations before engraftment (r=-0.09; P=0.63). In 6 patients whose peak BNP concentrations were ≥ 1000 pg/mL, cardiac performance was normal or slightly reduced (LVEF ≥50%) in 4 patients and moderately deteriorated (LVEF 30 - 50%) in 2 patients when they had peak plasma BNP concentrations. *Conclusions.* We found plasma BNP concentrations are elevated in patients with VOD and associated with the severity of VOD and mortality at day 100, irrespective of pretransplant cardiac function and coexistence of documented infection. These findings suggested plasma BNP may represent a prognostic marker of VOD and could offer a valuable tool toward therapeutic interventions.

0513

OUTCOME OF PEDIATRIC CD3/CD19 DEPLETED HEMATOPOIETIC STEM CELL TRANSPLANTS IN A SINGLE UK CENTER

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Background. T-cell depletion for allogeneic hematopoietic stem cell transplantation (HSCT), via graft manipulation, commonly involves CD34+ cell selection, rendering the graft deplete of CD34- cells. However, studies have suggested that cells lost through this method, particularly natural killer (NK) cells, play an important role in engraftment, immune reconstitution and the graft versus leukemia (GvL) effect. In response to this an alternative selection method has been developed involving $CD3^+/CD19^+$ cell depletion, thus retaining CD34- progenitors, NK cells and dendritic cells. *Aims.* This study reports a retrospective comparison of 59 $CD34^+$-selected pediatric HSCT's and 40 $CD3^+/CD19^+$ depleted pediatric HSCT's, in terms of engraftment, immune reconstitution and outcome. It is the first study to provide outcome data for patients receiving CD3+/CD19+ depleted grafts from both matched unrelated and haploidentical donors. *Methods*. All patients who underwent allogeneic HSCT at Birmingham Children's Hospital between January 2001 and October 2009 were identified retrospectively from local databases. Graft manipulation prior to April 2006 was $CD34^+$ selected; from May 2006 onwards grafts were $CD3^+/CD19^+$ depleted. All manipulated grafts were included in this study for analysis. Repeat transplants in single patients were excluded. *Results.* The study included 59 patients in the CD34 group and 40 patients in the CD3/19 group (median age 7 vs. 9 years), who underwent HSCT's for a variety of malignant and non-malignant diseases. The CD34 group included 6 matched related, 29 matched unrelated and 24 haploidentical transplants, whilst the CD3/CD19 group included 6 matched related, 23 matched unrelated and 11 haploidentical transplants. Groups were comparable based on age, diagnosis and graft type. Median follow up was 67 months in the CD34 group and 28 months in the CD3/CD19 group. Rejection rates were similar in the two groups, with 4 primary graft failures in each (P=0.826). Time to neutrophil engraftment (absolute neutrophil count>0.5×10^9/L) was identical, with a median of 12 days, but time to platelet engraftment (platelet count>50×10^9/L) was significantly shorter in the CD3/CD19 group (18 vs. 15 days, P=0.010). Time to immune reconstitution (CD3>0.3×10^9/L) was similar in the CD34 and CD3/CD19 groups (85 days vs. 100 days, P=0.269). Transplant related mortality under 100 days was similar (12% vs. 15%, P=0.650). There was significantly more acute graft versus host disease (GvHD) in the CD3/CD19 group (35% vs 55%, P=0.009) but no difference in chronic GvHD, veno-occlusive disease, or viral infection. Significantly, Kaplan Meier estimation of patients with malignant disease showed no difference in relapse rates between the two groups (28% vs 44%, Log Rank P=0.235). *Summary/Conclusions* In keeping with previous evidence there was a significant reduction in time to platelet engraftment in the CD3/CD19 group. However, we could not demonstrate a significant improvement in neutrophil engraftment or immune reconstitution. As expected higher rates of acute GVHD were seen in the CD3/CD19 group but this did not translate into improved relapse free survival. A randomized controlled trial is therefore necessary to further define the role of CD3/CD19 depletion in pediatric HSCT.

0514

EFFECT OF DONOR REGULATORY T CELLS ON CMV-SPECIFIC CD8+ T LYMPHOCYTES RECONSTITUTION AND ACUTE GVHD AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Backgound. Regulatory T-cells (T reg,$CD4^+CD25^{high}Foxp3^+$) are important regulators of allo-reactivity and may therefore represent an important predictor for the risk of acute graft versus-host disease (aGvHD) and immune reconstitution after allogeneic peripheral stem cell transplantation (allo-PBSCT). *Patients and Methods.* To determine the clinical significance of T regs in peripheral stem cell grafts, we analyzed 34 myeloablative alloPBSCT and correlated the T regs in the donor graft with the aGvHD incidence and immunological recovery (evaluated on recovery of CMV-specific $CD8^+$ T lymphocytes by tetramer analysis). We used fluorochrome-conjugated tetrameric complexes of HLA-A101, HLA-A201, HLA-B702, HLA-B801, HLA B3501 to monitor recovery of CMV-specific $CD8^+$ according to the patient's HLA. Patients were transplanted with unmanipulated peripheral blood stem cells from an HLA identical related donor (n=29) or an HLA identical unrelated donor (n=5). Median age was 32 years (range (r) 18-58); diagnoses were acute myeloid leukaemia (n=31) and acute lymphoblastic leukaemia (n=3). The median T regs dose administered was 5×10^6/Kg (r:1-20).The patients were divided into a high T regs group (T regs>5×10^6/Kg, n=14) and a low T regs group (T regs<5×10^6/Kg, n=20) according to the number of T regs in the grafts. *Results.* Median CMV-specific $CD8^+$ T lymphocytes were significantly higher in patients with high than with low T regs in the graft at 1(2 cells/mmc vs 0, P<.001), 2(6 cells/mmc vs1, P<.001), and 3(15 cells/mmc vs 3, P<.001) months. During the three months after transplantation, CMV infection/disease was observed in 2/13 (15%) patients with high T regs and in 12/20(60%) patients with low T regs (P=.015). Moreover, the median recovery of T regs after transplantation was significantly higher in patients with high T than with low T regs in the grafts at 2 (15 cells/mmc vs 6, P<.001) and 3 months(23 cells/mmc vs 8, P<.001). The incidence of aGvHD (grade II-IV) in the high infused T regs group was lower than in the low T regs group (1/13 or 7% vs 11/20 or 55%, P=.009). *Conclusions.* We suggest that there is a good correlation between the number of T regs in the graft and the incidence of aGvHD after myeloablative allo-PBSCT. T regs mediate protective effects against aGvHD and the maintenance of an optimal microenviroment for the reconstitution of functional immunity. Our results support further consideration of T regs immunotherapy for clinical alloSCT.

0515

IMPACT OF CYCLOSPORINE LEVELS ON THE DEVELOPMENT OF ACUTE GRAFT VERSUS HOST DISEASE IN THE REDUCED INTENSITY CONDITIONING ALLOGENEIC STEM CELL TRANSPLANTATION SETTING

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Background. Previous studies have supported that high serum cyclosporine (CsA) levels in the early phase after transplantation could result in a lower incidence of acute graft versus host disease (aGVHD) after myeloablative allogeneic stem cell transplantation (allo-SCT). If this finding also applies to reduced intensity allo-SCT (allo-RIC) has not been reported. *Aims.* The aim of the study was to analyze the impact of CsA levels in the development of grade 2-4 aGVHD (2-4 aGVHD) in the setting of allo-RIC in the first four weeks after transplantation. *Methods.* This study analyzes 134 consecutive patients [56 (41,8%) women], median age 53 (17-69) years old, who underwent HLA-identical sibling allo-RIC at a single institution. RIC included fludarabine 150 mg/m^2 plus busulphan (Bu) 10 mg/kg (for myeloid malignancies n=44 and PNH n=1) or melphalan (Mel) 70-140 mg/m^2 (lymphoid malignancies n=88 and aplastic anemia n=1). GVHD prophylaxis was based on CsA plus methotrexate (MTX) (n=113) or mychopenolate mofetil (MMF) (n=21). CsA levels were measured at least twice weekly during the first four weeks (or until discharge) and the dose was adjusted to maintain blood levels between 200 and 300 ng/mL. *Results.* As the use of MTX vs MMF and Bu vs Mel did not impact on the incidence of grades 2-4aGVHD patients were analyzed together. The median blood concentrations of CsA at 1st, 2nd, 3rd and 4th weeks after allo-SCT were 138 (range: 10-444), 214 (range:60-656), 247 (range: 53-586) and 224 ng/mL (range: 49-670) respectively. Forty patients (29,9%) developed grade 2-4 aGVHD for a cumulative incidence of 32% (95% CI 25-42%) at a median of 34 (range:18-137) days after allo-SCT. One hundred and seven (80%), 44 (33%), 25 (19%) and 19 (14%) of patients had levels below 150 ng/mL in the 1st, 2nd, 3rd and 4th week respectively. In univariate analysis the variables associated with a higher incidence of 2-4 aGVHD were: donor-recipient sex (female to male) P=0,05, CsA mean levels in the 3rd week (P=0.011), CsA levels below 150 ng/mL in the 3rd week (P=0,001) and CsA levels below 150 ng/mL in the 4th week (P=0,013). In multivariate analysis, the only significant variables associated with higher 2-4 aGVHD were CsA levels below 150 ng/mL in the 3rd (RR 2,6 (95% CI 1,2-5,6) P=0.014) and 4th weeks (RR 2.3 (95% CI 1-5.3) P=0.05). *Conclusions.* In the RIC setting, CsA level below 150 ng/mL in the third and/or fourth weeks after SCT is associated with a high risk of developing 2-4 aGVHD.

0516

THE ABSENCE OF KIR ACTIVATING RECEPTORS IN DONOR NATURAL KILLER CELLS INCREASES THE RISK OF ACUTE GVHD IN UNRELATED HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. NK cells exert their activity through interaction of Killer cell immunoglobulin-like receptors (KIRs) with specific ligands on the surface of target cells. Many of the KIRs recognize human leukocyte antigen (HLA) class I molecules. Two broad haplotypes of KIR genes have been defined. The A haplotype is characterised by the presence of a single activating KIR gene (2DS4), whereas the B haplotype is characterised by two or more activating KIR genes (2DS1, 2DS2, 2DS3, 2DS5 e 3DS1).). About 80% of homozygotes for KIR haplotype A carry the KIR2DS4*003 allele which is not expressed. *Aims.* The aim of this study was to evaluate whether the presence of the KIR2DS4*003 allele (not expressed) on donor KIR haplotype A could affect the outcome of unrelated hematopoietic stem cell transplantation (HSCT). *Methods.* We analyzed the KIR genotype profiles in 93 patients and their donors following allogeneic HSCT for thalassemia. All donor/recipient pairs were identical at molecular level for the HLA Class I and Class II loci. We also determined the allelic specificities of the activating KIR 2DS4 (*001, *002, *003). The conditioning regimen was the same in all patients. *Results and conclusions*. Out of transplanted patients, 70 are alive and well (disease-free survival 75%), 11 rejected and 12 died. Twenty-six patients (26/93-28%) developed acute graft-versus-host disease (aGVHD). In 9 of these patients, aGVHD was grade III-IV. Patients transplanted from donors with NK cells lacking activating KIRs (homozygous for KIR2DS4*003 which is not expressed) had a higher risk of developing aGVHD, particularly severe grade III-IV aGVHD (HR= 14.8; 95% CI: 2.8 - 78.5; P<.001). Overall, our findings suggest that the KIR gene profile and 2DS4 KIR expression should be included among the parameters used to predict the risk for severe aGVHD following unrelated HSCT.

0517

BETA-2 MICROGLOBULIN (B2M) IS A RELIABLE UNBIASED PREDICTOR MARKER FOR OUTCOME IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) AFTER ALLOGENEIC STEM CELL TRANSPLANTATION (ALLO-SCT)

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Background. Allo-SCT is the only treatment able to cure a fraction of patients with CLL. Advanced stage, extensive prior therapy, lack of response to therapy, and T cell depletion of the graft are considered to have a negative impact on outcome after allo-SCT. Nevertheless, to date there are no conclusive prognostic factors analysis to identify subgroup of patients who are most likely to benefit from being allografted. B2M has important prognostic value in patients treated with chemoimmunotherapy but has been scarcely investigated in the context of allo-SCT. Aims/*Methods.* We investigated the influence of prognostic factors including B2M in 50 patients who received an allo-SCT (32 non-myeloablative,18 myeloablative) in two institutions, between 1991 and 2008. Median age was 51 (29-63). Interval between diagnosis and SCT was 46 months (6-129). Median number of prior therapies, 3 (1-7). Eight patients had previously received an autologous SCT (auto-SCT). Most patients had adverse biologic features (high ZAP-70 expression, unmutated IGHV, poor cytogenetics). Serum B2M levels were ≥ 3.0 mg/L in 14 out of 48 patients prior to transplant. 48% of patients were refractory to fludarabine. Creatinine levels were normal. *Results.* Median follow-up after transplantation was 7 years (1.8-17). The relapse risk (RR) at 5 and 10 years was 15% (CI, 1-29) and 38% (CI, 11-65), respectively. At one and 10 years the non-relapse mortality (NRM) was 38% (95% CI, 25-51) and 43% (95% CI, 29-57). Five and 10-year progression free survival (PFS), event free survival (EFS) and overall survival (OS) were 85% (CI, 72-99) and 62% (CI, 35-89), 45% (CI, 30-60) and 32% (CI, 16-49), and 51% (CI, 37-65) and 48% (CI, 33-62). In the univariate analysis, factors associated with a higher NRM were prior auto-SCT (P=0.001), number of prior therapies (≤2 vs. >3) (P=0.04) and serum B2M levels (>3 mg/L) at the time of SCT (P<0.001). Time from diagnosis to SCT (>3 years) was associated with EFS (P=0.029) and high B2M levels (P<0.001), prior autologous SCT (P<0.001 and P=0.001), and number of prior therapies (≤ 2 vs. >3) (P=0.008 and P=0.023) were associated with both EFS and OS. In the multivariate analysis, prior auto-SCT was associated with EFS (RR=3, CI: 1.2-8.2; P=0.02) and OS (RR=3, CI: 1.1-7.7; P=0.03) whereas pretransplant B2M levels were an independent factor for NRM (RR=6.80; CI: 2.7-17), EFS (RR= 3.3; CI: 1.5-7.5; P=0.003) and OS (RR=4.4; CI: 1.9-10.3; P=0.001). Furthermore, B2M remained the strongest prognostic factor associated with outcome when the analysis was restricted to patients who received a non-myeloablative SCT. In contrast, other well known prognostic variables were not associated with outcome. *Summary.* Although B2M correlates with chemorefractory disease, this study indicates that, B2M is a strong predictor factor for outcome in CLL patients submitted to allo-SCT. Thus, whereas the diagnosis of refractory disease relies on criteria not always easy to be assessed (i.e, prior therapies, sites with remaining disease and their evaluation methods - clinical or imaging studies), B2M seems to be a reliable predictor factor of clinical outcome of patients with CLL receiving an allograft.

0518

EARLY VS CONVENTIONAL/LATE AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION IN MULTIPLE SCLEROSIS: LESSONS LEARNED OVER THE PAST TEN YEARS OF RUSSIAN-AMERICAN COOPERATION

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High dose immunosuppressive therapy with autologous hematopoietic stem cell transplantation (HDIT+AHSCT) is a new and promising therapy for severe autoimmune diseases which has been used with increasing frequency as a therapeutic option for multiple sclerosis (MS) during the last decade. Among a number of unclear questions is the timing for transplantation in MS patients. According to our concept there are 3 strategies of HDIT+AHSCT depending on terms of disease process: early (EDSS 1.5-3.0), conventional (EDSS 3.5-6.5) and late (EDSS 7.0-8.5). We aimed to study treatment outcomes in MS patients after early, conventional/late HDIT+AHSCT. 164 MS patients (secondary progressive - 57, primary progressive - 29, progressive-relapsing - 5, relapsing-remitting - 73) were included in this study (mean age - 33.0; male/female - 66/98). 72 patients underwent early transplantation, 84 - conventional, 8 - late transplantation. Median EDSS at baseline - 4.0. The mean follow-up duration - 22.9 months (range 6 - 128). Median follow-up duration - 20.6 months (range 6 - 128). Neurological evaluation was performed at baseline, at discharge, at 3, 6, 9, 12 months, and every 6 months thereafter. Transplantation procedure was well tolerated by the patients with no transplant-related deaths. The efficacy analysis was performed in 129 patients with the follow-up at least 6 months: 56 patients after early and 73 patients after conventional/late HDIT+AHSCT. At 6 months after transplantation the following distribution of patients according to the clinical response was observed: 26 patients (46%) achieved improvement and 30 patients (54%) stabilization after early transplantation; 35 patients (48%) demonstrated improvement, 37 patients (51%) - stabilization, and 1 patient (1%) - disease progression after conventional/late transplantation. At 12 months post transplant in the group of conventional/late HDIT+AHSCT one more patient progressed. No progression was registered in the group of early transplantation. At long-term follow-up (median - 24.7 months) after early HDIT+AHSCT improvement was observed in 17 (65%) patients, stabilization in 8 (31%), and progression in 1 (1%) patient. After conventional/late HDIT+AHSCT 20 (57%) patients improved, 11 (31%) were stable, and 4 (12%) progressed. No active, new or enlarging lesions were registered on MRI in patients without disease progression. Thus, HDIT+AHSCT appears to be a safe and effective treatment for MS. Early HDIT+AHSCT results in better treatment outcomes as compared to conventional/late transplantation. Further studies should be done to establish the best timing for HDIT+AHSCT.

0519

EFFICACY OF PLERIXAFOR PLUS G-CSF FOR STEM CELL MOBILISATION IN PATIENTS WITH MULTIPLE MYELOMA OR LYMPHOMA WHO HAVE FAILED PRIOR MOBILISATION - A NAMED PATIENT PROGRAM [NPP] EVALUATION

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Background. Growth factors alone or in combination with chemotherapy fail to mobilise adequate hematopoietic stem cells (HSC) for autologous transplantation in a substantial number of patients with multiple myeloma (MM) or lymphoma. Plerixafor, a CXCR4 receptor antagonist, was recently approved in Europe in combination with G-CSF for HSC collection in poor mobilisers. *Aims.* To evaluate the efficacy of mobilisation with plerixafor plus G-CSF in patients with MM or lymphoma who had failed prior mobilisation with G-CSF±chemotherapy. *Methods.* This was a retrospective analysis of patients in the NPP from 32 sites in the United Kingdom. Patients received G-CSF (10 µg/kg) each morning for 4 days. On the evening of Day 4, patients received plerixafor (0.24 mg/kg) by subcutaneous injection. On the morning of Day 5, G-CSF administration was followed by apheresis, occurring approximately 10 hours after plerixafor administration. Repeated administration of G-CSF and plerixafor was committed for up to 4 consecutive days, until adequate numbers of HSC were or were not collected.

Table. Summary of findings.

	MM n=84	NHL n=70	HD n=10	Other n= 2	Total n=166
Patients collecting >2 x 10^6 CD34+ cells/kg on Day 1 (%)	35 (41.6)	10 (14.3)	4 (40)	1 (50)	50 (30.1)
Patients collecting >2 x 10^6 CD34+ cells/kg in all apheresis days (%)	65 (77.4)	38 (54.3)	5 (50)	2 (100)	110 (66.3)
Patients who proceeded to transplantation, N (%)	63 (75)	36 (51.4)	7* (70)	1 (50)	107 (64.5)
Patients who achieved successful neutrophil engraftment (%)	62/63 (98.4)	35/36 (97.2)	7/7 (100)	0 (0)	104/107 (97.2)

**2 patients did not mobilize > 2 million CD34+ cells but proceeded to transplantation with cells pooled from other mobilisation attempts.*

Results. Of 253 patient requests complete data were available for 166 patients (MM, n=84, NHL, n=70, HD, n=10, other, n=2). The median age was 61 years (range, 24-70) and 89 (54%) patients were male. All patients had received chemotherapy (ranging from 1-9 regimens) prior to the remobilisation attempt with plerixafor; 46 patients had also received prior radiotherapy. Prior mobilisation history was available for 165 patients and included G-CSF alone in 22 (13%) patients, G-CSF plus chemotherapy in 125 (76%) patients, and both in 18 (11%) patients. The median peripheral blood $CD34^+$ cell count prior to plerixafor, available for 22 patients, was 5.5 $CD34^+$ cells/µL. Mobilisation with plerixafor plus G-CSF resulted in collection of the minimum target cell yield (>2×10^6 $CD34^+$ cells/kg) in 110 (66.3%) patients. The median number of apheresis days for all patients was 2 (range, 1-4). Of 166 patients, 107 (64.5%) proceeded to transplant. The median number of infused $CD34^+$ cells was 3.02×10^6 $CD34^+$ cells/kg (range, 1.6-8.8) The median time to neutrophil engraftment in 104 patients (MM, n=62, lymphoma, n=42) was 14 days(range, 11-32). Data on platelet engraftment were not collected. Follow up post transplant was available for 71 patients; at the date of last follow up 44 patients were in complete remission and 27 patients were in partial remission. There were 18 deaths (MM, n=5, lymphoma, n=13) in the total cohort of 166 patients (causes of death were disease progression, n=12; sepsis, n=2; and 1 each of gastrointestinal hemorrhage, pneumonitis, infective endocarditis, and cause unknown.) None of the deaths was deemed to be plerixafor-related. *Conclusions.* Mobilization with plerixafor + G-CSF achieved the target cell collection of 2×10^6 $CD34^+$ cells/kg in 110 (66.3%) of 166 patients who had failed prior mobilisation. To date 107 patients have been able to proceed to transplantation whereas this was not possible previously. These data are consistent with the US compassionate use program (Calandra 2007 BMT), further supporting the value of adding the CXCR4 antagonist plerixafor + G-CSF as a mobilisation agent.

0520

MONITORING OF MINIMAL RESIDUAL DISEASE BY QUANTITATIVE WT1 GENE EXPRESSION FOLLOWING REDUCE INTENSITY CONDITIONING ALLOGENEIC STEM CELL TRANSPLANTATION IN ACUTE MYELOID LEUKEMIA.

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WT1 is well-known to be a panleukemic marker that is expressed in 90% of acute myeloid leukemias (AML). Quantification of WT gene expression in bone marrow (BM) samples may be useful as a marker of minimal residual disease (MRD) during and after treatment for early prediction of relapse. We evaluated the validity of this AML-MRD marker after Reduce Intensity Conditioning (RIC) allogeneic Stem Cell Transplantation (SCT). The quantitative assessment of WT1 expression by Real-Time Quantitative PCR (RQ-PCR) was measured in 25 AML patients (pts) at diagnosis, at the time of RIC-SCT and after transplant at precise time points. All cases showed high WT1 levels at diagnosis with a mean of 4895 (SD 4462) and a median of 3679 (range 454-16853) copies WT1/104 ABL. At transplant 18/25 pts (72%) were in Complete cytologic Remission (CcR) and 7/25 (28%) had refractory AML. At the pre-SCT evaluation, bone marrow (BM) samples from pts transplanted in CcR showed significantly lower WT1 expression levels compared to the samples from pts with refractory AML (P=0.002). Median follow up after RIC-SCT was 18 months (range 2-54). On 18 pts transplanted in CcR, those (17/18) who maintained CcR after RIC-SCT displayed WT1 copy numbers persistently low during all the follow-up period. In patients who received RIC-SCT with active disease obtaining a sustained CcR after transplant (3/25), WT1 levels decreased to normal range in the first two months after RIC-SCT and remained low through the entire study period. All pts who relapsed after RIC-SCT (4/25) had a high WT1 copy number before the cytologic relapse. In 50% of these cases, an increase in WT1 expression was documented before molecular chimerism decreasing. With this experience, taking into account the limited number of pts, we confirmed a concordance between WT1 expression levels (measured by RQ-PCR at precise and sequential time points) and status of AML before and after RIC-SCT and we found a concordance between WT1 expression levels and hematopoietic chimerism status. Our data suggest that, in the RIC-SCT setting, the sequential and quantitative analysis of WT1 may be useful as a leukemia marker for monitoring MRD and as a predictor of overt AML cytologic relapse

0521

THERAPEUTIC POTENTIAL OF GAMMA DELTA T CELLS IN CONTROLLING CMV AND LEUKEMIA AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

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Background. Allogeneic stem cell transplantation (allo-SCT) is substantially hampered by GvHD, infections like CMV and relapse of disease. Gamma delta T cells (gdT cells) seem to be important in virus control but also in malignancy control by MHC independent recognition of antigens up regulated on stressed cells, however they do not mediate GvHD. In particular CMV infections are associated with an increased expansion of gdT cells, expressing the Vd1 chain. *Aims.* We investigated frequency and function of gdT cells after allo-SCT in order to assess their therapeutic potential. *Methods.*PBMCs at time points within 3 months after allo-SCT of 17 patients were sampled. CMV viral load was monitored by real-time PCR. Phenotype and frequency of gdT cells and alpha beta T cells (abT cells) were analyzed by flow cytometry. Two patients with CMV reactivation were selected and gdT

cells were isolated and expanded from time points before, during and after CMV reactivation. Two patients without CMV reactivation were used as controls. Frequency and clonality of $Vd1^+$, $Vd2^+$ and $Vd3^+$ gdT cells were measured with spectratyping. The reactivity against CMV AD169 infected fibroblasts, uninfected fibroblasts and primary AML blasts of $Vd2^-$ and $Vd2^+$ gdT cells was tested using an IFN-g ELISPOT. *Results.* We observed an increased polyclonal expansion of $Vd1^+$ gdT-cells after allo-SCT in patients during CMV reactivation and contraction after resolution as assessed by flow cytometry and spectratyping. Furthermore $Vd1^+$ but not $Vd2^+$ T-cells from these patients reacted against CMV AD169 infected fibroblasts, but not against uninfected fibroblasts. This suggests that $Vd1^+$ gdT-cells have the potential to dampen CMV infection after allo-SCT. Moreover, following pp65-reactive abT cells in HLA-$A2^+$ patients indicated that $Vd1^+$ gdT cells precede an abT-cell response. Finally, selectively $Vd2^+$ gdT cells had the potential to recognize primary leukemic blasts, but only in the presence of pamidronate, a phospho-antigen stimulating agent. *Conclusion.* GdT cells are present in patients after allo-SCT, have the potential to eradicate CMV infected fibroblasts and maybe the potential to spread an immune response to abT cells. Furthermore $Vd2^+$ gdT cells have the potential to recognize primary AML blasts. This strongly supports the idea to explore gdT cells as cell population for immune interventions after allo-SCT and the application of pamidronate post allo-SCT in patients suffering from AML.

0522

QUANTITATIVE MONITORING OF WT1 GENE EXPRESSION IN PERIPHERAL BLOOD AFTER ALLOGENEIC STEM CELL TRANSPLANTATION FOR ACUTE MYELOID LEUKEMIA - AN USEFUL TOOL FOR EARLY DETECTION OF MINIMAL RESIDUAL DISEASE

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Background. WT1 has been found overexpressed in the majority of patients with acute myeloid leukemia. *Aims.* Monitoring of the WT1 expression in peripheral blood (PB) may be useful as a marker of minimal residual disease (MRD) and may predict the relapse of AML after allogeneic stem cell transplantation (alloSCT). *Methods.* The quantitative expression of WT1 was measured in 40 patients with AML transplanted between 14.4.2005 and 18.11.2009. In 8 patients we had not data before alloSCT, three patients had not WT1 overexpression at diagnosis. For more precise analysis, we included a total of 29 patients (12 males, 17 females) who had significant WT1 overexpression at diagnosis or at relapse before alloSCT. The ELN recommended RQ-RT-PCR protocol was used for WT1 monitoring. *Results.* Overall 26 patients were transplanted in CR (5xCR1, 4xCR1 MRD+, 11xCR1 after second line treatment, 6xCR2), 3 patients were transplanted with active disease. Median follow-up after alloSCT was 17 months (range; 2-56). The level of WT1 expression correlated with attained or maintained CR in 19/27 patients. Eight (28%) patients relapsed after alloSCT after previous achievement of CR and all of them had an increase of WT1 expression at /or before relapse. Two patients transplanted with active disease died of progression without any response. At last follow-up: overall 6 patients died, 5 of relapse or progression, 1 of NRM. Four patients are treated for relapse and 19 patients remain in CR. In all cases, the increased WT1 expression correlated well with morphological examination, flow cytometry, chimerism and specific fusion genes. Moreover, in 4 cases, increased WT1 expression detected impending relapse sooner than chimerism , flow cytometry and morphological examination with median of 1 month (range 0,6-2,3) In 2 patients with the presence of specific fusion genes, their kinetics in relapse fully correlated with the WT1 kinetics. *Conclusions.* In our experience, there was a clear concordance between WT1 expression levels and AML status before and after alloSCT. WT1 could be useful as a non-specific leukemia marker for monitoring MRD and as a predictor of clinical relapse of AML. Monitoring of WT1 gene is probably most appropriate for patients transplanted in remission, where the increase in WT1 expression may signal a relapse in advance of standard investigative *Methods.* Based on these results, patients with increased WT1 levels after alloSCT and without graft vs. host disease may be candidates for immunosuppression tapering or DLI therapy .

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0523

CIK CELLS AND MESENCHYMAL STROMAL CELLS: A 'DANGEROUS' RELATIONSHIP

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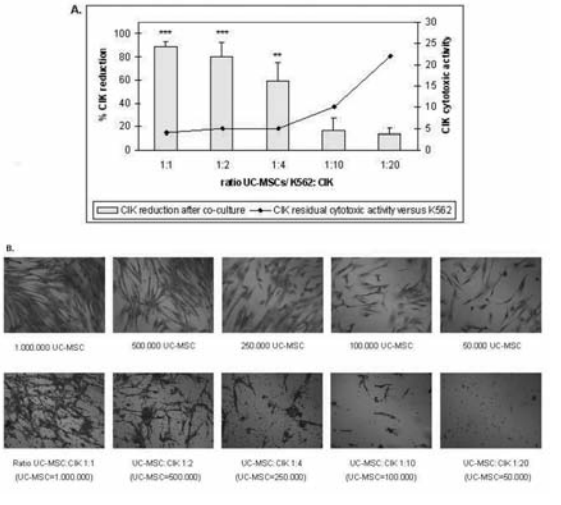
Background. Human mesenchymal stromal cells (MSCs) are multipotent cells possessing: self-renewal capacity; long term viability; multilineage potential and immunomodulatory properties. Cytokine-induced killer cells (CIK) are $CD3^+CD56^+$ T cells with natural killer-like cytotoxic activity against tumour cells of several lineages *in vitro*. *Aims.* In view of the potential role of MSCs and CIK cells in the immunotherapy of tumours, we explored the possible interactions between these two cell types. *Methods.* At the end of passage 3, umbilical cord-derived MSCs (UC-MSCs), cultured in DMEM supplemented with FBS (10%) and blocked with Mitomycin C (10 µg/mL), were co-cultured, at different ratios, with a constant number (1000000) of expanded CIK cells isolated from human peripheral blood of five healthy donors using Ficoll, and then stimulated with INFγ, OKT3 and IL2 for 14 days (UC-MSCs:CIK ratios: 1:1, 1:2, 1:4, 1:10, 1:20 and 0:1000000). The experiments were performed with the two cell types in contact or separated by trans-well membrane. After six days, CIK cells were gently resuspended, counted in a Burker chamber and tested for cytotoxic activity. UC-MSCs, exposed or not at CIK cells, were fixed, stained with haematoxylin-eosin and observed at microscopy. Residual cytotoxic activity of CIK cells was evaluated and quantified measuring the fluorescence of Calcein-AM released by K562, a chronic myeloid leukemia cell line, co-cultured for four hours with CIK cells at the following K562:CIK ratios: 1:1, 1:2, 1:4, 1:10, 1:20. *Results.* The apoptotic effect of UC-MSCs on CIK cells or vice versa was related to their ratio. At 1:1 ratio CIK reduction caused by UC-MSCs was of 88.4±4.4%; this percentage decreased in the presence of a diminishing number of mesenchymal cells (Figure 1A). On the other hand, decreasing their ratios, UC-MSCs were susceptible to lysis by CIK cells; at 1:20 ratio they were no more microscopically detectable (Figure 1B). Also CIK cells cytolytic activity against K562 was inversely proportional to UC-MSCs:CIK ratio, that is their cytotoxic effect increased when CIK were co-cultured in the presence of an decreased number of mesenchymal cells (Figure 1A line). All these apoptotic effects were related to cell contact; in fact they disappeared when the two cell lines were separated by trans-well membrane. *Conclusions.* These results should be taken into account when evaluating the combination of the two cell types in novel therapeutic strategies designed to improve engraftment, to suppress graft versus host disease or to exploit the effect of CIK-mediated graft versus leukemia. They suggest, for a future clinical application, to infuse before MSCs to support hematopoiesis and then CIK cells to kill residual neoplastic cells.

Figure 1. A. Reduction of CIK cells at different UC-MSC:CIK ratio (*** p<0.001; ** p<0.01 compared to CIK reduction at 1:20 ratio. n=5). CIK residual cytolytic effect against K562 after co-culture with mesenchymal cells (data referred to UC-MSCs:CIK ratio 1:1. Line). **B.** Haematoxilin-eosin stain showed that CIK cells were capable of killing MSC (magnification 100x).



0524

SIGNIFICANT REDUCTION OF MORBIDITY AND MORTALITY OF EARLY CMV DISEASE AFTER ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANTATION BY PRE-EMPTIVE THERAPY WITH ANTI-VIRAL DRUGS AND CMV CYTOTOXIC T LYMPHOCYTES

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Background. CMV disease is one of main causes of death after allogeneic hematopoietic cell transplantation (allo-HCT), especially from alternative donors. It is important to initiate pre-emptive therapy for CMV viremia since CMV disease usually has poor response to anti-viral medicines. *Aim.* In present clinical study, morbidity and mortality of CMV disease after pre-emptive therapy with anti-viral drugs and CMV specific cytotoxic T lymphocytes (CMV-CTLs) in allo-HCT recipients were investigated. *Methods.* From January 2007 to January 2009, total 334 patients who received allo-HCT were studied (matched sibling 100, unrelated 88, haploidentical146). The median age was 35 years (range, 5 to 55 years). Conditioning regimens were myeloablative with BUCY or CYTBI. ATG was added in unrelated and haploidentical transplants. CMV serological status of both donor and recipient before transplant was screened by ELISA. Plasma CMV DNA was monitored 1 to 2 times weekly with real time quantitative polymerase chain reaction (RQ-PCR). Ganciclovir was used for 8 days during conditioning. Either Ganciclovir or Foscarnet was used as front-line pre-emptive therapy when plasma CMV DNA turned to positive. Combination of Ganciclovir and Foscarnet or CMV-CTLs were administrated if the patients failed to front-line pre-emptive therapy. *Results.* Almost all recipients (100%) and donors (100%) were CMV serological positive. Overall 100-day cumulative incidence of CMV viremia was 69.1% (231/334) with median time of day 33 (range, day 11 to 79). Much lower incidence of CMV viremia was found in matched sibling transplant (33.3%) compared with unrelated (88.6%) and haploidentical (82.1%) transplants (P<0.001). Total 29.4% (68/231) patients received combined anti-viral medicines and 12.1% (28/231) patients were managed with CMV-CTLs with median cell dose 1.87×10^5 /kg (range, 2.4 103to 8.0×10^6/kg). CMV DNA became negative in 93.9% (217/231) patients after pre-emptive therapy. Fourteen patients developed CMV disease (enteritis 12 cases, interstitial pneumonia 1 case, encephalitis 1 case), which was much less than our historical control (Dao-Pei LU, *et al.* Blood 2006;107:3065). Overall 100-day cumulative incidence of CMV disease was only 3.8% although 70.1% patients underwent allo-HCT from either unrelated or haploidentical donors. There were no statistical difference in the incidences of CMV disease among transplants from matched sibling (3.0%), unrelated (4.5%), haploidentical (4.1%) donors (P=0.214). Only 5 patients died of CMV disease(they are all enteritis). No significant difference on 2-year overall survival was seen in patients with or without CMV disease (70.5% vs. 66.0%, P=0.397). Univariate and multivariate analysis showed that alternative donors and gradeII~IV acute GVHD were the risk factors for CMV reactivation. *Conclusions.* Viremia which is determined by RQ-PCR guided pre-emptive therapy with anti-viral medicines and CMV-CTLs significant reduces morbidity and mortality of early CMV disease in allo-HCT patients, even in the setting from alternative donors.

SIMULTANEOUS SESSION I

Myeloma and other monoclonal gammopathies - Biology

0525

CLINICAL OUTCOMES PREDICTION FOR NEWLY DIAGNOSED MULTIPLE MYELOMA PATIENTS TREATED WITH THALIDOMIDE-DEXAMETHASONE AND AUTOLOGOUS STEM CELL TRANSPLANTATION BY 8-GENE SIGNATURE OF CD138+ PLASMA CELLS

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Background. Efficacy of Thalidomide-dexamethasone (TD) as induction therapy in preparation for autologous stem cell transplantation (ASCT) in multiple myeloma provided the basis for the design of the phase II "Bologna 2002"study incorporating TD into double autotransplantation as up-front therapy for younger patients (pts) with newly diagnosed disease. *Aim.* We performed a molecular study aimed at identifying a gene expression profile (GEP) signature predictive of attainment of at least near complete response (nCR) to TD and subsequent autotransplantation. *Methods.* For this purpose, we analyzed bone marrow samples obtained at diagnosis from 112 pts who received TD before double ASCT. The differential gene expression of $CD138^+$ enriched plasma cells was evaluated by means of expression microarray using the Affymetrix platform. Significant expression results were validated by Real-time PCR. *Results.* Two subsequent study phases were planned. Firstly, a GEP supervised analysis was performed on a training set of 32 pts, allowing to identify 157 probe sets differentially expressed (P<0.05) in pts with at least nCR (e.g. responders) versus those who failed at least nCR (e.g. non responders) to TD induction therapy. Most of the 157 genes resulted down expressed in responder pts and were mainly involved in cell cycle and apoptosis regulation. In particular, signaling pathways which might be affected by the de-regulated expression of genes in responding pts, are the MAPK signaling pathway (Ppp3r1, PRKY, PRKX, FAS, ATF2, MAP4K3 and DUSP4), the Wnt signaling pathway (Ppp3r1, PRKY, PRKX and CCND2) and the p53 pathway (CCND2, FAS, CCNDE and MDM2).In the second phase of the study, we generated an 8-gene GEP signature which predicted at diagnosis the probability to achieve at least a nCR after TD induction therapy. The performance of this assay was subsequently validated by Real-time PCR in a training set of 80 pts: 36 pts were predicted as responders to TD , whereas 44 as non responders. The post autotransplantation outcome was analyzed according to Real-time expression results. On an intention-to-treat basis, the rate of CR, either immunofixation negative or positive, was 51.4% among CR-predicted ps and 23% (P=0-001) in the subgroup of 44 NR-predicted pts. The 65-months probability of OS for CR-predicted pts was 72% as compared to 41% for those who were predicted to be NR (P=0.03). The projected rates of TTP and PFS at 55 months for CR-predicted and NR-predicted pts were 69% vs. 34% (P=0.003) and 55% vs. 19% (P=0.01), respectively. *Conclusions.* These results can be an important first step to identify at diagnosis those pts who are more likely to respond favorably to a particular treatment strategy.

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0526

MICRORNAS CHANGES OCCUR IN MULTIPLE MYELOMA CELLS IN THE CONTEXT OF BONE MARROW MILIEU

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Introduction. It has been previously demonstrated that primary multiple myeloma (MM) cells are characterized by a specific microRNA (miRNA) signature compared to the related normal plasmacell counter-

part; and that miRNAs play a crucial role in regulating MM pathogenesis. Nevertheless, miRNA changes that occur in MM cells in the context of the bone marrow microenvironment have not been previously examined. *Aims.* To evaluate miRNA signature in MM clonal plasmacells. To evaluate the role of bone marrow microenvironment in regulating miRNA signature in MM clonal plasmacells. *Methods.* miRNA expression profiling has been performed on MM cell lines (MM.1S, RPMI) either alone or in co-culture with bone marrow stromal cells (BMSCs); MM primary CD138$^+$ bone marrow-derived CD138$^+$cells; healthy subjects bone marrow-derived CD138$^+$ cells. *in vitro* and *in vivo* functional studies were performed on miRNA-15a- and -16-1-precursors-transfected MM cells. Effect of miRNA-15a and -16-1 on signaling cascades have been evaluated by western blot and immunofluorescence. *in vivo* MM cell growth has been evaluated by either using an *in vivo* imaging model or bioluminescence. Angiogenesis has been studied both *in vitro* and *in vivo* using the chorioallantoic membrane model. *Results*. miRNA-15a and -16-1 are down-regulated in MM cells as compared to normal cells. Precursor-miRNA-15a- and -16-1-transfected cells showed decreased DNA synthesis; decreased cyclinD1/cyclinD3/cdk6/pRb protein expression; phase G1 cell cycle arrest; as compared to either scramble probe-transfected or not transfected MM cells. Moreover, transfected cells showed inhibition of NFkB pathway as shown by reduced p65-/p50-/p52-NFkB activities; downregulation of p-p65/p50/p52 nuclear protein level; upregulation of phospho-IkB in the cytoplasm; and inhibited translocation of p-p65 from the cytolplasm to the nucleus. Similarly, inhibition of MM cell growth was confirmed *in vivo*; and anti-angiogenic properties of miRNA-15a and -16-1 were validated both *in vitro* and *in vivo*. miRNA profiling of MM cells cultured with primary BMSCs (MM+BMSC system) differs from MM cells which were not grown in contact with primary BMSCs (MM cells alone). Specifically, we observed increased expression of miRNA-450/-432*/-299-5p/-409-3p/-29b/-542-5p/-184/-517*/-218/128b/-142-5p/-211 (P<0.05) in MM cells obtained from the MM+BMSC system, compared to MM cells alone. Stem-loop qRT-PCR was performed on matched samples and showed expression patterns similar to those observed in miRNA analysis. Predicted human miRNA gene targets of the increased miRNAs included negative regulators of NFkB, PI3K/Akt/mTOR, and MAPK/ERK signaling pathways (PTEN/KSR2/TWEAK/DUSP); as well as tumor suppressors (MCC/TSSC1/TUSC1/FBW7/RHOBTB), pro-apoptotic factors and cyclin-dependent kinases inhibitors. *Summary.* These data demonstrate that miRNAs play an important role in MM pathogenesis by regulating plasmacell tumor clone growth; and that bone marrow stromal cells exert a modulatory effect on miRNA profiling in MM cells, which results in promoting MM cell growth and inducing MM cell survival.

0527

GENETIC VARIATIONS ASSOCIATED WITH TREATMENT RESPONSE, PROGRESSION FREE SURVIVAL AND OVERALL SURVIVAL IN PATIENTS WITH MULTIPLE MYELOMA IN THE HOVON 50/GMMG-HD3 TRIALS

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Background. Thalidomide improves overall response and quality of response before and after high dose melphalan in patients with multiple myeloma (MM). In addition, it prolongs progression free survival (PFS), but not overall survival (OS), due to inferior survival from relapse. Genetic instability and clinical variables (ISS) are prognostic variables for outcome. Little attention has been given to structural host factors as additional markers to predict treatment response and survival. Single nucleotide polymorphisms (SNPs) may influence may influence the individual response to treatment and could therefore act as an independent class of predictors. *Aims.* The aim of this study was first to determine if genetic variations are associated with response to therapy and survival in patients with multiple myeloma and second to analyse these effects in thalidomide based versus traditional vincristine/adriamycin/dexamethasone (VAD) based induction treatment. *Methods*. We collected peripheral white blood cell DNA from 532 MM patients included in the Dutch-German HOVON-50/GMMG-HD3 clinical trials comparing 3 cycles of VAD, to thalidomide 200 mg orally, days 1 to 28 plus adriamycin and dexamethasone (TAD) as induction regimens (Lokhorst HM. *et al.*, Blood. 2010 Feb 11;115:1113-20). Genetic variations were analyzed using a custom-built molecular inversion probe (MIP) based SNP chip, designed by 'Bank on a Cure' (BOAC), containing 3404 SNPs selected in "functional regions"within 983 genes representing cellular functions and pathways that may influence disease response, toxicities, complications, and survival. A Cochran-Armitage trend test was performed to determine SNP associations with response to therapy, PFS and OS for VAD and TAD treated patients separately using the program PLINK. To account for multiple testing we carried out label swapping procedures. *Results.* The results of the SNP association analyses for response to TAD and VAD treatment both show associations with the ADME genes (drug absorption, distribution, metabolism, and excretion) ABCC1 and ABCC2. In addition, only TAD response is associated with ABCB11 (P=.002) and ABCC4 (P=.01). Inflammatory genes are associated with TAD response, whereas genes involved in cell death are associated with VAD response. In TAD treated patients, significant associations for both PFS and OS were observed with SNPs located in the DNA repair genes LIG3 (P=.003 and P= .002 respectively) and SHFM1 and the inflammatory genes TNF, SELE (P= .002 and P=.03 respectively) and F13A1. Furthermore, SNPs associated with PFS were located in the ADME genes CYP39A1 (P=.002), ABCC2 (P=.002) and CYP2F1. *Summary/Conclusions.* In this study we identified SNPs which were associated with treatment response, overall and progression free survival in multiple myeloma patients receiving TAD or VAD induction treatment. These results indicate that variations in drug metabolism, inflammatory response and DNA repair are crucial in treatment outcome. We conclude that SNPs contribute to treatment outcome and survival.

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0528

THE CLINICAL AND BIOLOGICAL SIGNIFICANCE OF THE IMMUNOPHENOTYPIC ASSESSMENT OF CD81 IN MULTIPLE MYELOMA CLONAL PLASMA CELLS

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Background. Although CD19 is typically down regulated in myelomatous plasma cells (MM-PC), we have recently shown that a minority of multiple myeloma (MM) patients (4%) express this marker at diagnosis, which correlates with adverse outcome. The CD19 expression is thought to be regulated by CD81, a tetraspanin involved in mechanisms of cell proliferation. However, phenotypic or genomic studies of CD81 expression in MM are scanty, and its potential prognostic value remains unknown. *Aims.* To assess the frequency and the prognostic value of the immunophenotypic detection of CD81 surface expression in MM-PC of smoldering and symptomatic MM patients at diagnosis. *Methods.* A total of newly diagnosed 36 smoldering MM (SMM) patients and 229 symptomatic MM patients were included in this study, the latter group uniformly treated according to the Spanish GEM05>65y protocol. Expression of CD81 on MM-PC was assessed by multiparameter flow cytometry (MFC), staining BM samples using a four-color direct immunofluorescence technique that allowed the identification of MM-PC as well as CD81 surface expression. In a subset of patients (18 SMM, 23 MM) mRNA gene expression profiling (GEP) was performed on immunomagnetically enriched MM-PC. *Results.* MFC studies detected positive staining for CD81 in MM-PC of 15/36 (42%) SMM and 90/229 (39%) MM patients. Interestingly, both SMM and MM CD81$^+$ cases showed a higher frequency of CD19 expression on MM-PC compared to CD81- cases (13% vs. 0%, P=.08 and 7% vs. 1%, P=.01; respectively), in line with the regulatory role of CD81 over CD19. Concerning GEP analysis, we found a significantly (P=.003) lower relative expression of CD81 mRNA in MM-PC of SMM (6.8) and MM (6.7) patients compared to normal PC (9.3), which could explain, at least in part, the absence of CD81 on MM-PC surface in ≈half of myeloma cases. Accordingly, we found a signifi-

cant correlation between GEP and MFC expression of CD81 (r=.743; P<.001), and CD81+ SMM and MM patients showed higher levels of relative expression of CD81 mRNA compared to CD81- cases (7.5 vs. 6.6, P=.03 and 7.4 vs. 6.7, P=.04, respectively). No significant differences were found in baseline characteristics of CD81- vs. CD81+ SMM or MM patients, except for the% of MM-PC in S-phase (0.8 vs. 1.4, P=.09 and 0.9 vs. 1.4, P=.003 for SMM and MM, respectively). Finally, CD81+ SMM patients had a shorter time to progression to symptomatic disease than CD81- cases (NR vs. 25 months, P=.04, Figure A); and also CD81+ MM showed significantly lower response rates (complete remission: 8% vs. 26%, P=.01), progression-free (23 months vs. NR, P=.001, Figure B) and overall survival (P=.03, Figure C) than CD81- cases. In MM patients, a multivariate analysis identified two baseline variables with independent prognostic value for PFS: highly abnormal sFLC ratios (<0.02 or >10;HR=3.0;P=.008) and CD81+ expression (HR=2.2; P=.02) , whereas for OS only CD81+ expression (HR=2.3;P=.05) was selected. *Conclusions.* Our findings uncover the existence of a phenotypic/genomic correlation of CD81 expression in MM-PC, which correlated with an adverse outcome in SMM and MM, supporting the clinical relevance of baseline routine BM evaluation by MFC in myeloma.

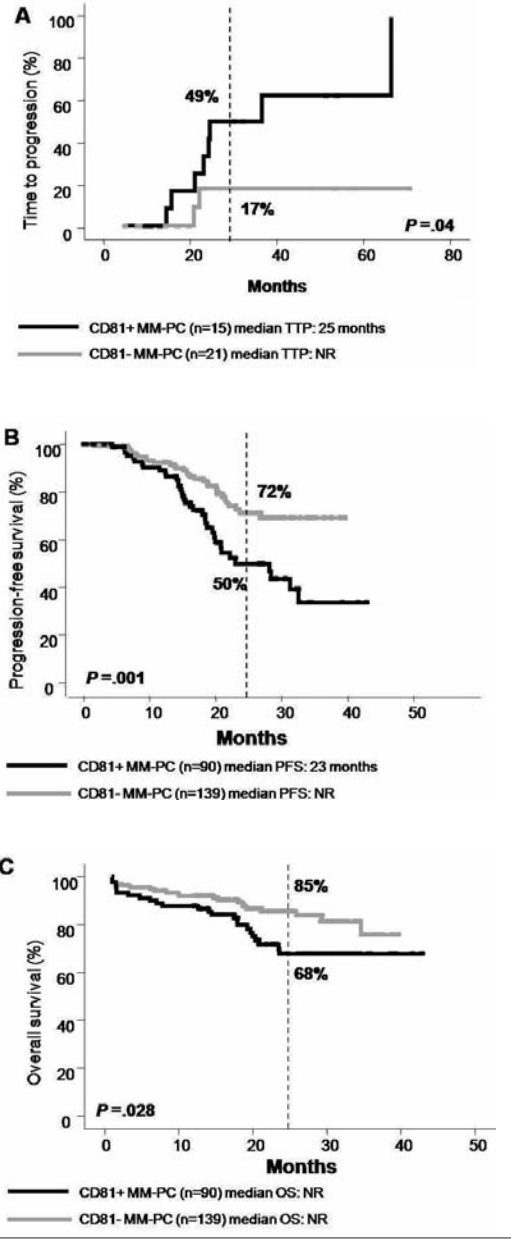

Figure.

0529

DEVELOPMENT OF PERIPHERAL NEUROPATHY IN MULTIPLE MYELOMA PATIENTS; INCIDENCE, MOLECULAR CHARACTERIZATION AND EFFECT ON RESPONSE IN BORTEZOMIB VS CONVENTIONAL TREATMENT

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Background. The proteasome inhibitor bortezomib has demonstrated high antitumor activity in multiple myeloma (MM). Bortezomib induced peripheral neuropathy (BiPN) is a major dose-limiting toxicity. Chemotherapy induced peripheral neuropathy has also been described with thalidomide and vincristine in MM. The exact mechanisms of both BiPN and vincristine induced peripheral neuropathy (ViPN) are not fully understood. *Aims.* The aim of this study was to gain more insight in the mechanism underlying BiPN and ViPN in MM patients and to identify differences in incidence and to unravel underlying molecular characteristics. In addition we evaluated the association of BiPN with response to therapy. *Methods.* Genetic variation associated with BiPN or ViPN was analyzed using a custom-built molecular inversion probe (MIP) based single nucleotide polymorphism (SNP) chip, designed by 'Bank on a Cure' (BOAC), containing 3404 SNPs selected in "functional regions"within 983 genes representing cellular functions and pathways that may influence disease response, toxicities, complications, and survival. MM samples were taken from the Dutch-German HOVON-65/GMMG-HD4 clinical trial comparing standard induction treatment with vincristine combination (VAD) to bortezomib combination (PAD) prior to high" dose therapy (HDT) and stem cell transplantation. For BiPN 51 cases and 80 controls and for ViPN 20 cases and 107 controls were included in a SNP association analysis. Samples from 329 MM patients from this trial were analyzed for tumor gene expression using U133 2.0 arrays to gain more insight in the characteristics of myeloma plasma cells at baseline which could play a role in the development of BiPN or ViPN, and to differentiate between early or later development of BiPN. Cox regression analysis with BiPN as a time-dependent covariate was used to analyze the prognostic value of BiPN on achieving at least a very good partial response (VGPR). *Results.* The observed associated SNPs with BiPN differ considerably from those associated with ViPN. SNPs associated with BiPN were located in the DNA repair genes BRCA1 and ERCC4 (P=.0004) and in inflammatory genes F2 and F13A1. SNPs associated with ViPN were mainly located in genes involved in cell death such as JUN (P=.001) and the metabolism genes ABCC4 (P=.008) and FMO3. The genetic profile of myeloma plasma cells from patients developing BiPN included genes involved in immune cell trafficking, such as PIK3CG, MFHAS1, SOX8, RAPH1 and CKM1 (FDR<.05). Upregulated genes in patients with ViPN included interferon induced genes, such as IFIT2, IFIT3, CXCL10 (FDR<.05), and the inflammatory gene TNFSF13B (FDR<.05). BiPN grade 2-4 was observed in 27% of patients during induction treatment and was significantly associated with achievement of VGPR and CR with PAD, hazard ratio (HR)=1.42, 95% CI=1.03-1.97 (P=.033). *Summary/Conclusions.* In this study we showed unique genetic associations for BiPN and ViPN, indicating different mechanisms may be involved in the development of BiPN and ViPN. Development of grade 2-4 BiPN was significantly associated with achievement of VGPR and CR on induction with 3 cycles of PAD. Our results contribute to a better understanding of the mechanisms underlying BiPN and ViPN and differences between these two types of peripheral neuropathy.

Thrombosis and Bleeding

0530

PREVALENCE AND DETERMINANTS OF BLEEDING IN SEVERE VON WILLEBRAND DISEASE TYPE 3: RESULTS OF RETRO/PROSPECTIVE STUDIES IN A COHORT OF 105/52 ITALIAN PATIENTS

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Background. von Willebrand disease type 3 (VWD3) is due to virtually complete deficiency of the von Willebrand factor (VWF) and, for this reason, has been also described as "severe VWD". Although rare (1-5 cases per million population), VWD3 is of major interest because of its severe clinical presentation, the need for replacement therapy with VWF/FVIII concentrates and the risk of occurrence of anti-VWF inhibitors after the infusion of VWF/FVIII concentrates. *Aims.* and design of the study: to determine the prevalence and determinants of bleedings requiring therapy with VWF/FVIII concentrates in VWD3 patients, data were collected from the Italian registry on Hemophilia and Allied Disorders . Some of these VWD3 patients were then followed up for one year by six Italian Centers and prospective data on number, type and management of bleeding episodes were analyzed. *Patients and Methods.* VWD3 patients were diagnosed when VWF antigen was undetectable and factor VIII (FVIII) levels were reduced in plasma. Bleeding severity score (BSS) was calculated at enrollment. Gene deletions and mutations were searched for in all available DNA. Bleeding-free survival was computed with the Kaplan-Meier method and a Cox's proportional hazard model was used to calculate the risk of bleeding (hazard ratio = HR). *Results.* In the Italian registry, 105 VWD3 patients (5.7%) were identified among the 1850 VWD (Italian prevalence=1.75 per million). The entire cohort of VWD3 was characterized by the following demographic, clinical and laboratory parameters (median, range): gender (M/F)= 50/55; age=37 (3-65); BSS=18 (3-35); FVIII= 4 (2-18); anti-VWF inhibitors= 7 cases (6.7%) from 3 families. Molecular diagnosis was available in 65/105 cases with the following gene defects (pt-n): large deletion (7); small deletions and insertions (23); nonsense (9); splice site (8) and missense mutations (17). Mucosal bleedings (64%) were more frequent than hematomas and hemarthrosis (24%). At the time of the enrollment in the registry 95/105 (91%) VWD3 had been already exposed to VWF/FVIII concentrates because of bleeding and/or minor or major surgeries. In the prospective study, 52 VWD3 patients could be enrolled and 46 (88%) were treated in a year for 118 bleeding episodes and 27 minor or major surgeries. BSS>10 (6.8, 3.8-12.3) and FVIII<10U/dL (4.1, 2.4-7) were significantly associated with high risk of bleeding. The bleeding-free survival at one year calculated according to values of BSS and FVIII levels showed 4 different KM curves with the following *Results.* 89% (BSS=5-10&FVIII>10U/dL); 64%(BSS>10&FVIII=5-10U/dL); 59% (BSS=5-10&FVIII<10U/dL); 18% (BSS>10&FVIII<5U/dL). VWD3 patients were given 192 injections of VWF/FVIII concentrates for bleeding and surgeries. Patients with anti-VWF inhibitors were off therapy during this follow-up. *Conclusions.* these retro/prospective studies performed for the first time in a relative large cohort of patients show that also VWD3 can be very heterogeneous. BSS and FVIII levels are good predictors of bleeding. Multicenter studies with larger number of cases should be organized to identify additional modifiers of bleeding risk in VWD3

0531

PLATELET DYSFUNCTION AND MDS FEATURES DUE TO NOVEL SOMATIC GATA1 MUTATION

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Background. Germline exon 4 mutations in the N-terminal zinc finger (N-finger) of GATA1 cause X-linked dyserythropoiesis with macrothrombocytopenia, while a germline splicing mutation in exon 2 leads to congenital neutropenia and anemia due to exclusive expression of GATA1s, the short isoform of GATA1. Acquired GATA1 exon 2 splicing mutations occur in both transient myeloproliferative disorder (TMD) and acute megakaryoblastic leukemia (AMKL) in Down syndrome patients. *Aims.* In the present study, we describe the functional characteristics of a somatic GATA1 mutation (E200G) in a boy with a lifelong history of bleeding problems, anemia, neutropenia, severe bone pains and hepatosplenomegaly, and bilineage dysplasia in the bone marrow. *Methods.* Platelet morphology and function was studied. Different *in vitro* and ex vivo experiments were applied to study the functional effect of the GATA1-E200G mutation. *Results.* The patient has a clinical bleeding problem, an increased Ivy bleeding time and a prolongation of the PFA100 occlusion time. Electron microscopy revealed normal sized platelets with few dense granules. Platelet ATP secretion and aggregation were decreased. The a to g substitution was found in GATA1 mRNA (exon 4) from platelets and bone marrow mononuclear cells, and in genomic DNA from purified myeloid progenitors, but not lymphoid progenitors, lymphocytes and fibroblasts. This GATA1-E200G mutation affects a highly conserved residue at the beginning of the N-finger, but does not perturb DNA binding to GATA sites and partially reduces its interaction with FOG1. In addition, the mutation is located at the exonic splice site and is associated with alternative splicing and reduced GATA1 protein levels in *in vitro* differentiated megakaryocytes from $CD34^+$ hematopoietic stem cells isolated from peripheral blood of the patient and a control subject. *Conclusions.* This study expands the GATA1 phenotype-genotype spectrum with the development of MDS and platelet dysfunction due to a somatic GATA1 splice mutation.

0532

ANNEXIN II IS OVER EXPRESSED IN HCG-PML-RARα LEUKEMIC MICE AND IS ASSOCIATED WITH HIGHER LEVELS OF TISSUE PLASMINOGEN ACTIVATOR (TPA)

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Background. Acute Promyelocytic Leukemia (APL) is frequently associated with a coagulation abnormality that is the main cause of morbidity in this disease. At least three components can be indentified: activation of coagulation cascade, proteolysis and fibrinolysis. Annexin II, a receptor for both tissue plasminogen activator (tPA) and plasminogen has been shown to be over expressed in human APL and may be involved in fibrinolytic abnormalities. The murine model of APL (hCG-PML-RARα) evolves to acute leukemia in about 15%, and cells from leukemic mice can be transplanted. Few is known from coagulation in this model and plasmine activator Inhibitor (PAI) and tumor necrosis factor α (TNFα) appear to be elevated. *Aims.* We aimed to compare Annexin II expression in a murine transplant model of APL as well as plasmine and tPA plasmatic levels. *Methods.* We studied two groups of wild type mice that received each 200-cGy irradiation followed by infusion of 2×10^6 cells from bone marrow of wild type animal (group Wild) or from a leukemic hCG-PML-RARα animal (group Leu). On day 21 after transplantation the animals were sedated with tribhromoethanol followed by inferior vena cava infusion of sodium citrate and blood collection. Plasma was separated and analyzed for tPA and plasmine by ELISA method. Cells from experimental animals bone marrow was obtained through infusion of RPMI in large bones and analyzed by flow cytometry to quantify Annexin II and CD117 expression. We used Mean Fluorescence Channel (MFC) ratio and D value (Kolmogorov-Smirnov statistics) to estimate Annexin II expression. *Results.* Mean MFC ratio was 1,038 in group Wild and 1,702 in group LEU (P<0,001) and D value was 0,0938 and 0,9380 (P<0.001), respectively. D value in CD117 positive cells was also different between groups (0,1163 in group Wild and 0,396 in group Leu). We also observed a correlation (Pearson coefficient: 0,734- P=0.001) between CD117 positive Cells and Annexin II expression. tPA levels were elevated in Leu group (12,14% versus 10,71%, P<0.001) but no difference was observed in plasmine levels (233,4 mg versus 233,8 mg). There was also a correlation between Annexin II expression (both MFC ratio and D value) and tPA values (Pearson coefficient=0.913 for MFC ratio and 0,833 for D - P<0.001). *Conclusions.* We confirmed that, similar to human APL, bone marrow cells from hCG-PML-RARα leukemic mice over express Annexin II. We also observed higher levels of tPA, but no difference was seen in plasmine levels, which may be due to its half-life. The correlation of Annexin II and tPA levels suggests that this protein may actually be involved in fybrinolytic abnormalities seen in APL. This murine model is adequate to explore Annexin II and fibrinolytic pathways interactions.

0533

FREQUENCY, TYPES AND MANAGEMENT OF BLEEDING EPISODES IN ACQUIRED VON WILLEBRAND SYNDROME: A FIVE-YEAR PROSPECTIVE STUDY IN A COHORT OF 18 PATIENTS WITH CHRONIC LYMPHO-MYELOPROLIFERATIVE DISORDERS (LPD/MPD)

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Background. Acquired von Willebrand Syndrome (AVWS) is a rare bleeding disorder with laboratory findings similar to those of inherited VWD. Actual prevalence, frequency and types of bleedings of patients with AVWS are unknown because large prospective studies are not available. AVWS is frequently associated with monoclonal gammopathy of uncertain significance (MGUS) or Essential Thombocythemia (ET). *Aims.* To determine frequency and types of bleeding episodes which might need therapeutic interventions we have prospectively followed-up for five years our cohort of AVWS *Patients and Methods.* 18 patients were enrolled in the study on January 2005 with diagnosis of AVWS performed according to the criteria of ISTH-SSC. A bleeding severity score (BSS) was calculated after exposing patients at enrollment to a detailed questionnaire. Bleeding time (BT) and VWF activities were measured in all cases while platelet nucleotides only in ET. Bleeding-free survival was computed with the Kaplan-Meier method. Types and number of treatments needed to manage bleeding were also recorded. *Results.* AVWS associated with MGUS (n=10) showed higher mean values of BSS than those (n=8) with ET (15 versus 6) in agreement with lower mean levels of VWF:RCo (8 versus 35 U/dL) and FVIII (12 versus 65 U/dL). Among the 8 cases with ET, BSS was relatively higher when low VWF:RCo levels were associated with lower platelet nucleotides. BT was moderately prolonged, with values of 16 (MGUS) and 12 (ET) min. The bleeding-free survival at five year calculated according to MGUS and ET was 20% and 50%, respectively. Mucosal (n=36) and non-mucosal (n=23) bleeds in MGUS (case n=8) or ET (case n=4) were treated with DDAVP (n=38), VWF concentrates (n=43), IVIg (n=26), rFVIIa (n=12). *Conclusions.* Patients with AVWS associated with MGUS/ET can show severe bleeding requiring intensive treatment. An early correct diagnosis should improve morbidity and mortality of these patients.

0534

IDIOPATHIC VEIN THROMBOSIS: IDENTIFICATION OF POPULATIONS AT DIFFERENT RISK OF RELAPSE FOLLOWING ORAL ANTICOAGULANT TREATMENT. THE RESULTS OF THE 'EXTENDED-DACUS STUDY'

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Background. The safest duration of anticoagulation, after the first episode of idiopathic Deep Vein Thrombosis (DVT), is unknown. *Aims.* We evaluated, in a prospective management study, the optimal Vitamin K-Antagonist (VKA) duration with reference to the risk of thrombosis relapse according to Residual Vein Thrombosis (RVT). *Methods.* Patients with a first idiopathic DVT were evaluated for the presence of RVT after 3 months of VKA; subsequent thrombosis and/or bleeding events were recorded during treatment and during the one-year follow-up after VKA withdrawal.

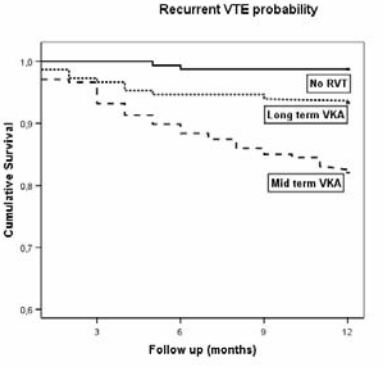

Figure 1. Kaplan-Meier curves of recurrent VTE after VKA sus.

Results. In 29.9% of patients, no RVT was found after 3 months of therapy and VKA was stopped; the remaining patients received anticoagulants for additional 15 or 21 months. Among the RVT-negative group, the incidence of recurrent VTE and major bleeding was 1.2% (2/164) and 0. Among the RVT-positive groups, the incidence of thrombosis was 7% (27/384) while on anticoagulation. After VKA withdrawal, the incidence of recurrences was 17.9% in the mid-term and 6.7% in the long-term group (Figure 1). Major bleeding was observed in 1% (4/384) of the cases. *Conclusions.* Our results indicate that in patients without RVT, a short VKA treatment is sufficient; in those with persisting RVT, treatment duration extended to 2 years substantially reduces but not abolishes, the risk of thrombosis recurrence.

Myelodysplastic syndromes

0535

PROPOSAL OF A NEW, COMPREHENSIVE CYTOGENETIC SCORING SYSTEM FOR PRIMARY MDS

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Background. The IPSS-Score, published by Greenberg et al. (1997), defines the gold standard in risk stratification of patients with MDS. Since its implementation in 1997 based on 816 patients with primary MDS, the knowledge concerning the prognostic impact of distinct abnormalities increased extensively. *Aims.* The present study proposes a new and comprehensive cytogenetic scoring system based on an international data collection originating from the German-Austrian (GA)-, the International Risk analysis workshop (IMRAW)-, the Spanish Cytogenetics working group (GCECGH) and the International Cytogenetics Working Group of the MDS Foundation (ICWG). *Methods.* Inclusion criteria were defined as follows: Primary MDS, age >=16, and bone marrow blasts <=30%. Regarding therapy, exclusively patients with supportive care were included. Based on these criteria, 955 patients were excluded resulting in 2901 patients available for final analysis. Univariate and multivariate analysis concerning overall survival (OS) and AML-transformation (AML-t) was performed. Age, gender, bone marrow blast count, number of peripheral cytopenias, center and year of diagnosis were included in the multivariate model. OS and AML-t in distinct cytogenetic abnormalities was only calculated when the abnormality occurred as an isolated aberration with a minimal frequency of n=10. Median observation time was 19.0 (0.1-326) months. Clinical follow-up was performed until April 2009. *Results.* In total, 20 cytogenetic subgroups were included, allowing the prognostic classification of 92% of all patients. Abnormalities were grouped as normal (n=1543, 53.2% of all cases), single (1 abnormality), double (2 abnormalities) or complex (>=3 abnormalities). Single abnormalities found were: der(1;7) (10, 0.4%); der(3)(q21)/der(3)(q26) (10, 0.4%); del(5q) (180, 6.4%); -7/7q- (60, 2.1%); +8 (133, 4.8%); del(11q) (20, 0.7%); del(12p) (18, 0.6%); i(17q)(11, 0.4%); +19 (10, 0.4%); del(20q) (48, 1.7%); +21 (10, 0.4%); -Y (60, 2.1%) and any other single (154, 5.5%). Double abnormalities were stratified into 3 subgroups: double including del(5q) (45, 1.6%); double including -7/7q- (31; 1.1%) and any other double (98, 3.4%). Complex karyotypes were sub-divided into 2 groups: Karyotypes with 3 abnormalities (60, 2.1%) vs. >3 abnormalities (196, 7.0%). 21 pts. (0.7%) displayed cytogenetically unrelated clones. According to OS and AML-t, abnormalities were classified to 5 prognostic subgroups: very good (del(11q), -Y), good (normal, der (1;7), del(5q), del(12p), del(20q), double incl. del(5q)); intermediate (-7/7q-, +8, i(17q), +19, +21, any other single, any other double, independent clones); poor (der(3)(q21)/der(3)(q26), double incl. -7/7q-, complex 3 abnormalities) and very poor (complex >3 abnormalities). Median survival was 60.8 months for very good (n=80), 48.5 months for good (n=1844), 24.0 months for intermediate (n=578), 14.0 months for poor (n=101) and 5.7 months for very poor (n=196); P<0.0001. Multivariate analysis resulted in a Hazard Ratio of 1.0 for very good (reference category), 2.1 for good, 3.4 for intermediate, 6.0 for poor and 9.3 for very poor concerning OS (p <0.0001). *Summary/Conclusions.* We were able to generate a solid database for a revised cytogenetic scoring system, which can serve as the cytogenetic model for the upcoming revision of the IPSS. *Acknowledgements.* The authors like to thank the MDS-Foundation for its support.

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DETECTION OF RARE ABNORMALITIES AND KARYOTYPE EVOLUTION IN MDS PATIENTS FROM PERIPHERAL BLOOD: FIRST RESULTS FROM THE MULTICENTER GERMAN PROSPECTIVE DIAGNOSTIC CD34+FISH-STUDY

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Background. In myelodysplastic syndromes (MDS) chromosomal aberrations can be detected in 50-80% of patients (pts). They play an important role for pathogenesis, prognosis, diagnosis and, more recently for treatment allocations. The acquisition of clonal abnormalities in pts with initially normal karyotype, the expansion of an aberrant cell clone with a given anomaly or the occurrence of new, secondary abnormalities are called karyotype evolution (KE). A model of stepwise cytogenetic changes is proposed, but only a few systematic studies had focused on this phenomenon. In MDS most chromosomal anomalies detected by banding analyses of bone marrow (bm) metaphases are provable by fluorescence *in situ* hybridisation (FISH) of bm as well as by enriched $CD34^+$ stem cells from peripheral blood (pb). *Aims.* In a multicenter German diagnostic study we analyse immunomagnetically enriched circulating $CD34^+$ cells in MDS pts by FISH using 2 different FISH probe panels for initial screening and sequential follow-up measurements to detect chromosomal aberrations in pb and follow the clone size during therapy. Methods CD34+ stem cells from pb are enriched by immunomagnetic cell sorting (MACS®) and analysed by FISH afterward using a "Superpanel"(D7/CEP7, EGR1, CEP8, CEP XY, D20, p53, IGH/BCL2, TEL/AML1, RB1, MLL, 1p36/1q25, CSF1R, all Abbott® Products) for initial screening, after 12 and 24 months and in every case of suspected disease progression and a "Standardpanel"(EGR1, D7/CEP7, CEP8, p53, D20, TEL/AML1, CEP X/Y) every 2 months during the first year and every 3 months during the second year. *Results.* After 17 months of study time 142 pts (78 m, 64 f) with suspected or cytomorphologically proven MDS from 10 German centres of haematology/oncology are included in our study: With regard to age, gender distribution and MDS subtypes the study cohort is representative for the disease. The median follow-up time is 4.6 months (1-15 months). In 77 pts (54%) chromosomal aberration were detected by FISH of circulating $CD34^+$ cells: 43 pts (56%) with one isolated anomaly, 15 pts (19%) with 2 anomalies, 10 pts (13%) with 3 aberrations and 9 pts (12%) with more than 3 anomalies. In decreasing frequency we detected del(5q) (n=37), del(7q)/-7 (n=25), allelic loss of TP32 (n=12), del(12p) (n=11) and del(20q) (n=10) as the most commen aberrations. In 12 pts of 99 with at least 2 analyses (12%) KE could be detected by FISH from pb. In 5/12 cases KE was followed by cytomorphological progression to higher stages of MDS. Discussion KE is a known phenomenon in MDS suspected to lead to increased chromosomal instability and a stepwise progression of the disease. The frequency of KE in our cohort is comparable to other studies published in literature. Our results show that close-meshed sequential FISH analyses of circulating $CD34^+$ cells are a feasible and sensitive method to monitor MDS pts. KE can be diagnosed early in the course of disease, so it might help to learn more about rare abnormalities and clonal evolution in MDS.

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PROLIFERATIVE AND APOPTOTIC SIGNALLING IN BONE MARROW CELL SUBPOPULATIONS OF MYELODYSPLASTIC SYNDROMES PATIENTS USING FLOW-CYTOMETRY TECHNIQUE

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Background. Myelodysplastic syndromes (MDS) are heterogeneous clonal diseases characterised by cytopenias as a result of ineffective

hemopoiesis. Development of effective treatments has been mainly impaired by limited insights into MDS pathogenesis. Moreover, little is known about signal transduction pathways altered MDS cells. *Aims and Methods.* We have devised a multiparameter flow-cytometry method of signal transduction pathways analysis allowing rapid and specific separate evaluation in specific cellular subpopulations of MDS bone marrow cells. Cells were fixed with formaldehyde and permeabilized with methanol, then stained with APC anti-human CD34, PE anti-human CD71, PerCP anti-human CD45 and Alexa-Fluor488 anti-STAT5 (pY694), Alexa-Fluor488 anti-ERK1/2 (pT202/pY204), Alexa-Fluor488 anti-p38 (pT180/pY182) and Alexa-Fluor488 anti-cleaved caspase-3 (D175). Samples were analysed on a cytometer with 6 color laser. We studied in 60 MDS cases phosphorylation of ERK1/2 and p38 MAP kinases, phosphorylation of signal transducer and activator of trascription (STAT)5 and proteolitic activation of caspase-3 in bone marrow mononuclear cell subpopulations $CD34^+$, $CD45^+$ and $CD71^+CD45^-$ and compared with normal bone marrow cells. We performed the analysis before and after erythropoietin (EPO) and granulocyte colony stimulating factor (G-CSF) stimulation. *Results.* Baseline activation of MAPK phospho-proteins, STAT5 and caspase-3 was variable among MDS cases and among distinct cellular subpopulations. Non-parametric Wilcoxon test indicated that STAT5 was significantly activated in all $CD34^+$, $CD45^+$ and $CD71^+CD45^-$ MDS cells compared with cells from normal controls while caspase-3 was activated in $CD34^+$ and $CD45^+$ MDS cells. By the Kruskal-Wallis test we observed that refractory anemia (RA) and refractory anemia with excess of blasts-1 (RAEB-1) were more prevalently affected by this phenomenon. G-CSF stimulation activated STAT5 in $CD34^+$ from MDS and normal controls, without significant differences. EPO stimulation failed to induce STAT5 activation in $CD71^+CD45^-$ cell subpopulation of 22/36 MDS cases while it was effective in normal cells and in 14/36 $CD71^+CD45^-$ MDS cells. Non-parametric Wilcoxon test showed that STAT5 activation induced by EPO in $CD71^+CD45^-$ MDS cells was significantly lower than in normal cells. Evaluation of EPO response *in vivo* (Hb increase > 2 g/dL without transfusions after 8 weeks of EPO treatment) has shown that in 20/22 cases it correlated with *in vitro* EPO dependent STAT5 activation (Spearman's rho=0.62 and P=0.002). *Conclusions.* Signal transduction pathways may be analysed separately in specific subpopulations of MDS marrow cells. Our study indicates that different subpopulations ($CD34^+$, $CD45^+$, $CD71^+CD45^-$) show diverse pathway activations and diverse apoptosis pattern. Response to growth factors is also restricted to specific cellular subpopulations.

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AZACITIDINE TREATMENT PATTERNS, HEMATOLOGIC IMPROVEMENT, AND TOLERABILITY IN A LARGE GROUP OF ELDERLY PATIENTS WITH MYELODYSPLASTIC SYNDROMES (MDS) IN THE AVIDA REGISTRY TREATED IN A COMMUNITY SETTING

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Background. Treatment decisions for elderly patients with MDS remain challenging, with little clinical data from controlled studies for guidance. Elderly patients are typically under-represented in clinical trials, although in higher-risk MDS patients ≥75 years of age in the AZA-001 study (N=87), azacitidine (AZA) was shown to significantly prolong survival compared with conventional care regimens (Seymour, Blood 2008:A3629). AVIDA is a large, prospective, US, community-based registry of patients treated with AZA. This registry includes the largest cohort of lower-risk (IPSS risk Low or Int-1) MDS patients treated with AZA. *Aims.* We evaluated AZA treatment patterns, hematologic improvement (HI), and tolerability in patients aged ≥75 years in the AVIDA registry. *Methods.* These interim data were collected between Oct 2006 and Nov 2009. Data were collected at registry entry, then quarterly, via electronic data capture. Treating physicians decided AZA dose, dosing schedule, and treatment duration. Reported AZA dosing schedules included 7 consecutive days, 5-2-2 (5 dosing days, 2-day break, 2 additional dosing days), and 5 consecutive days. HI and transfusion independence (TI) were assessed centrally using IWG 2000 criteria. Adverse events (AEs) were graded per NCI-CTCAE v3.0. *Results.* 434 patients ranging in age from 29 to 91 years were in the registry at the time of this analysis, Nov 12, 2009. Of them, 226 (52%) were age ≥75 years (mean [±SE] 80.8±0.3), half of whom (n=128, 57%) were ≥80 years of age. For these 226 patients, average time since MDS diagnosis was 15.3±2.0 months, 76% had baseline ECOG performance status 0-1 and 24% had ECOG PS ≥2, and of patients with IPSS data, 68% had lower-risk (IPSS 0-1) and 32% had higher-risk (IPSS ≥1.5) MDS. Patients received a median of 4 AZA cycles (range 1-24), with 38% receiving at least 6 cycles with a median follow-up of 5.0 (range: 0.03-25.0) months. Half of all treatment cycles (51%) were administered ≤28 days after the previous cycle, 29% occurred at 29-35 days, and 19% occurred at ≥36 days. For patients with available data, the most common route of AZA administration was intravenous (59%) vs subcutaneous (41%). More than half of patients (53%) received a 5-day AZA course, 16% received 5-2-2 dosing, and 13% received 7 consecutive day dosing. Approximately two-thirds of patients achieved HI or RBC TI (Table). Grade 3-4 hematologic AEs included neutropenia (14%), anemia (12%), and thrombocytopenia (12%); most occurred during cycles 1-2 and decreased in frequency thereafter. Grade 3-4 infections occurred in 13% of patients and predominately included pneumonia (5%). Patients <75 years of age had comparable effectiveness (Table) and safety outcomes. *Conclusions.* These AVIDA registry data, from the largest cohort of lower-risk MDS patients treated with AZA, show that among patients aged ≥75 years, AZA is preferentially dosed on a 5-day schedule, with approximately half of all AZA cycles administered without delay. These results also validate the effectiveness of AZA in patients with lower- or higher-risk MDS aged ≥75 years treated in a community setting, and demonstrate that AZA yields high rates of HI and RBC TI and is generally well-tolerated.

Table.

IWG-2000 Response	Patients receiving AZA Age ≥75 years (N=197*)	Patients Receiving AZA Age <75 years (N=185*)
Hematologic Improvement†		
Any HI	118/194 (61%)	98/174 (56%)
HI-Erythroid (Major)	82/181 (45%)	76/163 (47%)
HI-Platelet (Major)	51/117 (44%)	37/101 (37%)
HI-Neutrophil (Major)	19/98 (19%)	19/111 (17%)
Transfusion Independence‡		
Red blood cells	69/103 (67%)	60/92 (65%)
Platelets	19/26 (73%)	18/30 (60%)

*Ongoing patients with <56 days on-study were not eligible for analysis
†Individual cell line denominators include only patients eligible for improvement
‡ Denominators include only patients who were baseline transfusion-dependent (ie, received at least 1 transfusion in the 56 days prior to the start of AZA dosing).

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GENETIC TYPING OF CBL, ASXL1, AML1, TET2, AND JAK2 IN JUVENILE MYELOMONOCYTIC LEUKEMIA (JMML) REVEALS A GENETIC PROFILE DISTINCT FROM CHRONIC MYELOMONOCYTIC LEUKEMIA (CMML)

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Background. JMML and CMML are rare myeloproliferative and myelodysplastic neoplasms occurring at both ends of life, in infancy and in the elderly respectively. They share several diagnostic criteria. *Aims.* In order to better understand the pathophysiological relationship between JMML and CMML, we screened mutations of genes recently involved in CMML in a series of patients with JMML. *Methods.* 65 JMML have been studied. Mutations in *NRAS, KRAS, NF1* or *PTPN11* were present in 17%, 15%, 5% and 45% of cases respectively. The presence of germline mutations has been verified using fibroblasts as a source of constitutional DNA. Mutations in CBL (*c-CBL*), ASXL1, AML1 (*RUNX1*), and *TET2* have been screened by direct bi-directional sequencing. *JAK2*^{V617F} has been searched by TaqMan® probe hybridization. 51 JMML have been studied by SNP-array (Affymetrix SNP 6.0). *Results.* The mutation frequency in genes associated with JMML (*RAS, PTPN11* and *NF1*) or CMML (*CBL, ASXL1, AML1* and *TET2*), has been assessed in 65

patients with JMML and compared with that reported in CMML. Homozygous mutations of CBL associated with 11q23-qter copy neutral loss of heterozygosity were found in 4 out of 65 patients (6%). None of these patients had mutation in *RAS, PTPN11 or NF1*. A p.F418L substitution was found in one case and a p.Y371H substitution in the 3 others. These 3 latter patients presented with mild developmental anomalies, which led us to suspect the presence of an underlying genetic condition. A germline heterozygous mutation of CBL was found in these three patients. The mutation was *de novo* in 2 patients and inherited from the father in the third case. Two heterozygous mutations of *ASXL1*, p.G645VfsX58 and p.G646WfsX12 were found in 2 out of 65 patients (3%). Both mutations lead to a truncated *ASXL1* protein, which is removed from its PHD finger at the C-terminal extremity. These mutations have been identified in 2 patients older than 4 years at diagnosis and carrying a mutation in *NRAS* or *PTPN11*. No mutation in *TET2, AML1*, and *JAK2*^{V617F} genes was found in our series of JMML although these genes are mutated in 42%, 26% and 7% of CMML respectively. SNP-array pangenomic analysis did not show any abnormalities at these loci in JMML. *Summary/Conclusions.* JMML and CMML present mutations in CBL but substitutions found in JMML seem to be specific and are germline in a subset of children, thus defining a new predisposing syndrome to JMML. Among the various genes recently implicated in CMML, only *ASXL1* was found mutated in JMML. *ASXL1* belongs to the polycomb family gene and is a regulator of chromatin remodeling. Frameshift mutations lead to a truncated protein, which compromise the function of the associated chromatin modifiers. *ASXL1* mutations may be associated with JMML progression rather than initiation. In conclusion, the pattern of genetic lesions observed in JMML differs from that of CMML and confirms the crucial role of RAS and growth factor signaling deregulation in JMML.

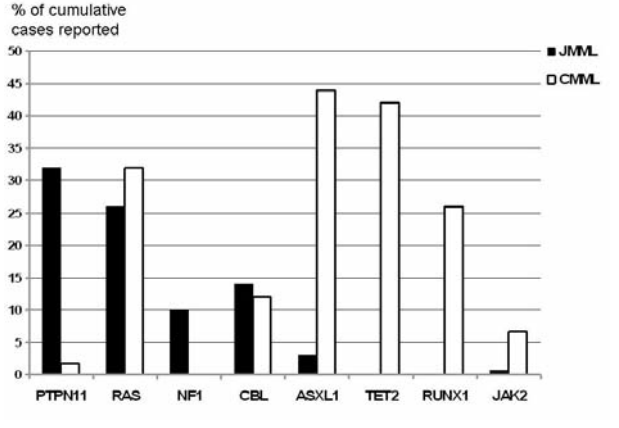

Figure 1. Genetic typing of JMML versus CMML

Chronic lymphocytic leukemia

0540

HS1 HAS A CENTRAL ROLE IN LEUKEMIC B CELLS TRAFFICKING AND HOMING

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Background. In Chronic Lymphocytic Leukemia (CLL) the relationships between cell accumulation within lymphoid organs and proliferation are not elucidated, and the mechanisms regulating CLL cell migration and re-circulation between peripheral blood and the lymphoid tissues are poorly characterized. Our previous studies demonstrated that Hematopoietic cell specific Lyn substrate 1 (HS1), a pivotal molecule in the signal transduction pathway triggered by the B-cell receptor, is a potential prognostic marker in CLL and interacts with several cytoskeletal components. Even though HS1 function in normal and leukemic B cells is poorly defined, both our data and the dissection of the protein structure prompted us to hypothesize an actin-binding activity as well as a potential role in cytoskeletal organization. *Aims.* Given the role played by the cytoskeleton in controlling cellular shape, mobility and homing, we hypothesized that HS1 could be potentially relevant in the regulation of CLL cells infiltration into lymphoid tissues and re-circulation between peripheral blood and tissues. *Methods.* To study HS1 function, we silenced its expression in a CLL cell line (MEC1) using an RNA Interference approach, and we utilized B lymphocytes from HS1 Knockout (KO) mice. Both cell types were then studied for their migration, adhesion, actin-polymerization and aggregation capacity by *in vitro* and *in vivo* assays. *Results.* In both cellular systems HS1-deficient B cells were severely impaired in their spontaneous migration and adhesion capacity. A decrease in F-actin polymerization and an increased homotypic aggregation ability were also evident in cells lacking HS1. Moreover, in the absence of HS1, B cells failed to form acto-myosin complexes and showed a defect in actin distribution and filopodia formation. To test *in vivo* HS1 function, we injected MEC1 cells silenced for HS1 subcutaneously in Rag2-/-γc-/- mice and observed that cells lacking HS1 spread and localize preferentially in the bone marrow (BM) and in the lymph nodes as compared to control cells. To further investigate HS1 role in the onset and progression of CLL, we crossed HS1 KO mice with Eμ-TCL1 transgenic animals (CLL mouse model). The HS1 KO/TCL-1 transgenic mice showed an earlier disease onset and a preferential accumulation of leukemic cells in the BM where they are usually observed only at low frequencies in the Eμ-TCL1 mouse. *Conclusions.* These findings suggest that HS1 has an important role in controlling cell migration and tissue invasion by leukemic B cells, likely through its involvement in cytoskeleton organization. This points at HS1 as a potential target for development of novel cancer treatments aimed at interfering with tissue infiltration and invasion.

0541

THE GENETICS OF RICHTER SYNDROME IDENTIFIES DISEASE HETEROGENEITY AND IS AN INDEPENDENT PREDICTOR OF SURVIVAL POST TRANSFORMATION

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Background. A fraction of CLL transforms to Richter syndrome (RS),

generally represented by diffuse large B-cell lymphoma (DLBCL). Knowledge of the genetic lesions driving RS transformation is scant. Aim. We aimed at exploring the pattern and prognostic impact of RS molecular lesions. *Methods.* Upon pathological review, all RS (n=81) were classified as DLBCL. Candidate genetic lesions were selected among those recurrently affecting *de novo* DLBCL and CLL. Mutational analysis of TNFAIP3/A20, CARD11, BLIMP1, CD79A/CD79B, EZH2, BCL6, c-MYC, PAX5, TTF, PIM1 and TP53 was performed by Sanger sequencing. Probes used for FISH were: i) LSI13, LSID13S319, CEP12, LSIp53, LSIATM, LSI IGH/BCL2, LSI BCL6, LSI IGH/c-MYC/CEP8, c-MYC breakapart, LSI N-MYC; BCL3 split signal; iii) 6q21/alpha-satellite; iv) BAC clones 373L24-rel and 440P05-BCL11A. Clonal relationship between CLL and RS was assessed by immunoglobulin gene analysis. Informed consent was obtained. *Results.* MUM1 was expressed in 77.3% RS, BCL6 in 25.4%, CD10 in 5.6%. IG were unmutated in 65.4% cases. TP53 mutations occurred in 28/80 (35.0%) RS, CARD11 mutations in 5.3%, BCL6-intron1 mutations in 7.1%, c-MYC mutations in 3.6%, PIM1 mutations in 10.7%, PAX5 mutations in 7.1%, and TTF mutations in 14.3%. Mutations of TNFAIP3/A20, BLIMP1, BCL6-exon1, CD79A/CD79B, and EZH2 were consistently absent in RS. c-MYC activation occurred in 25.9% RS (translocation: 13.8%; amplification: 12.1%), BCL2 amplification in 10.5%, BCL6 amplification in 7.0%, and BCL6 translocation in 1.8%. 17p13 deletion occurred in 27.1% RS, 11q22-q23 deletion in 16.0%, +12 in 12.1%, 13q14 deletion (MIR15/16B) in 13.7%, 6q21 deletion in 8.3%, MYCN amplification in 3.8%, and BCL3 translocation in 2.1%. TP53 disruption by mutation and/or deletion occurred in 48.3% cases.

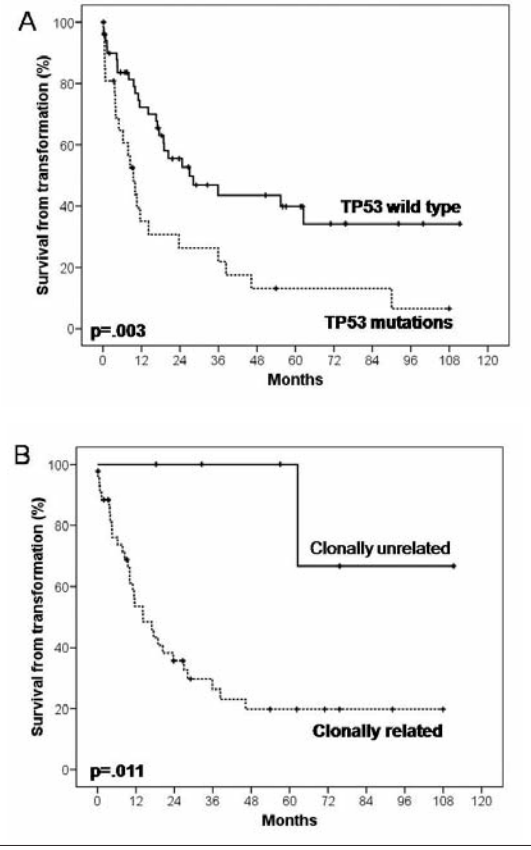

Figure 1.

Analysis of paired clonally related CLL/RS showed that TP53 disruption, c-MYC activation and CARD11 mutations are acquired at transformation. We then investigated the impact of molecular lesions on RS survival. At diagnosis, 64.1% RS were older than 60 years, 35.9% showed ECOG PS >1, 75.6% Binet stage B-C, 93.6% Ann Arbor stage III-IV, 43.6% B symptoms, 44.9% tumor size >5 cm, 30.8% involvement of >1 extranodal site, 62.5% LDH elevation, 69.2% Hb <11 g/dL, and 30.8% platelets $<100+10^9$/L. Biological variables associated with poor RS survival were TP53 mutation (P=.003), del17p13 (P<.001), and MUM1 expression (P=.047) (Figure 1A). Multivariate analysis selected TP53 mutations (HR:1.86 P=.040) as an independent predictor of RS survival along with ECOG PS (P=.001), tumor size (P=.001), and platelet count (P=.049). Clonally unrelated RS (7/81; 8.6%) differed from clonally related RS (45/81; 55.6%) because of lower prevalence of MUM 1 expression (33.3% vs 82.2%; P=.010) and TP53 mutations (0 vs 47.7%; P=.033). This difference translates into longer survival of clonally unrelated RS compared to clonally related RS (5-years survival: 66.7% vs 19.8%; P=.011) (Figure 1B). *Conclusions.* This study documents that: i) the genetic pattern of RS differs from that of *de novo* DLBCL; ii) TP53 disruption and c-MYC activation are the most frequent RS genetic lesions; iii) TP53 mutations are an independent prognostic factor in RS; iv) clonally unrelated RS are biologically and clinically distinct from clonally related RS.

0542

SELECTIVE INHIBITORS OF THE SPLEEN TYROSINE KINASE BLOCK CELL SURVIVAL AND MIGRATION IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. There is growing evidence suggesting that B-cell receptor (BCR) signaling plays an important role in the pathogenesis of chronic lymphocytic leukemia (CLL). BCR signaling in CLL cells activates the spleen tyrosine kinase (Syk), which leads to a number of downstream events that promote cell survival. Therefore, disrupting BCR-induced signaling by inhibiting Syk represents a novel therapeutic approach in CLL. A recent clinical trial indicated that inhibition of the Syk by R788 (fostamatinib disodium, FosD) is effective in patients with CLL and other B cell malignancies. R788 is a relatively selective Syk inhibitor, but also displays activity against Flt3, Jak, and Lck. *Aims.* Since Syk has been implicated in CLL cell activation, migration and survival, the present study was designed to determine the effects of BCR stimulation and its inhibition with two selective Syk inhibitors. *Results.* Pre-treatment with selective Syk inhibitors (P142-76 and P505-15) and a Syk/JAK inhibitor (P420-89), abrogated the increased CLL cell migration and survival of CLL cells in response to BCR engagement. Activation of the BCR with polyclonal goat F(ab')2 fragments to human IgM significantly increased CLL cell viability compared to control, and this pro-survival effect of BCR triggering was abrogated by treatment with the Syk inhibitors P142-76 and P505-15. The mean relative CLL cell viability at 48 hours was decreased to 78±4% (P142-76), 62±5% (P505-15) or 50%±4.5% (P420-89) of controls (100%; data are means±SEM, n=19). Furthermore we found that the inhibitors induce apoptosis in CLL cells in co-culture with nurselike cells (NLC), indicating that Syk inhibition antagonizes microenvironment-derived survival signals from NLC.

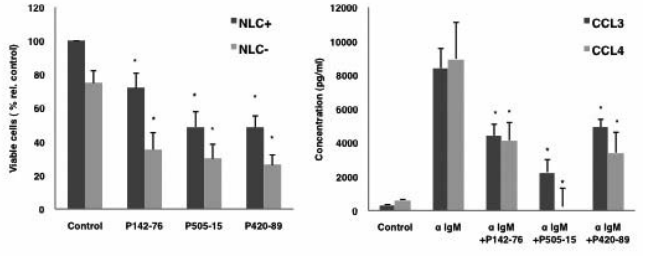

Figure. Syk inhibitors block BCR-induced CLL cell survival.

As shown in Figure A, the selective Syk inhibitor P505-15 significantly reduced CLL cell viability in NLC co-cultures from 84.4± 5% to 46±8% at 48 hours and the Syk/JAK inhibitor P420-89 to 52± 6% (mean ± SEM, n=6,*P<0.05). We recently reported that BCR triggering enhances CLL cell chemotaxis toward CXCL12 and CXCL13 (Blood 114:1029-37, 2009). To determine the effects of the selective Syk inhibitors on chemotaxis, CLL cells were incubated with anti-IgM with or without the Syk inhibitors before the chemotaxis assay. The selective Syk inhibitor P505-15 decreased chemotaxis toward CXCL12 and CXCL13 to levels that were 49.5% ± 5% or 32.8±6% of respective controls. Engagement of BCR induces the secretion of the chemokines CCL3 and CCL4 by CLL cells, which was almost completely abrogated by inhibiting Syk. As displayed in Figure B, BCR triggering induced CCL3 supernatant levels of 8400 pg/mL ± 1166 pg/mL and CCL4 levels of 8959 pg/mL ± 2147 pg/mL (mean ± SEM, n=5). Preincubation with P505-15 significantly reduced the levels of these chemokines to 2263 pg/mL ± 744 pg/mL (CCL3) and to 2250 pg/mL ± 1093 pg/mL (CCL4), respectively (mean ± SEM, n=5, *P< 0.05). This inhibition of CCL3/4 secretion could also be

demonstrated in CLL cocultures with NLC. *Conclusion*. In this study we demonstrate that inhibition of Syk with selective, small molecule inhibitors is highly effective in disrupting different BCR-derived responses. Specifically, these selective inhibitors antagonize CLL cell survival after BCR triggering, and in suspension- and NLC-cultures. Moreover, they inhibit CLL cell migration towards the chemokines CXCL12 and CXCL13. Also, they inhibit BCR- and NLC-induced secretion of the chemokines CCL3 and CCL4 by CLL cells. These results support the future therapeutic development of these selective Syk inhibitors in patients with CLL.

0543

IMPACT OF TP53 MUTATIONS ON OUTCOME: RESULTS FROM THE CLL8 TRIAL (FC VS. R-FC) OF THE GCLLSG

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There is growing evidence that TP53 mutations are associated with poor survival in CLL. There are limited prospective trial data on treatment effects of chemoimmunotherapy in CLL with and without TP53 mutation. The current study set out to define the impact of TP53 mutation in patients treated with FC or R-FC in the CLL8 trial. Analysis of TP53 mutation status by microarray-based re-sequencing assay (Amplichip p53 assay in development by Roche Molecular Systems) and confirmatory direct DNA sequencing were performed in a central reference laboratory. Samples were available for 628 (76.9%) patients at study entry and this cohort was representative of the full trial population regarding other baseline prognostic factors and demographics. In addition, 94 follow-up samples of 89 patients at relapse were available. The incidence of the TP53 mutations was 11.9% (71/628; 41 in FC arm, 30 in R-FC arm). Forty-two of 51 patients (82.4%) with 17p deletion had a TP53 mutation. 5% of patients without 17p deletion (28/553) had a TP53 mutation. Patients with TP53 mutation showed lower complete response (CR) and overall response (OR) rates as compared to the group without TP53 mutation (6.9 vs. 36.4% and 62.1% vs. 95.3% (P<0.001). Lower response rates were observed for the TP53 mutation groups in both arms (FC and R-FC): CR (3.2% and 11.1%), CR+PR (51.6% and 74.1%). Response rates for patients without TP53 mutation were 24.2% (CR (FC)) and 92.2% ORR (FC) vs. 47.8 (CR R-FC arm) and 98.2% (ORR R-FC). Median progression free survival (PFS) was significantly shorter for patients with TP53 mutations (12.3 months vs. 45 months) (HR: 4.4 (3.29-5.87) P<0.001). Median PFS was better for patients in the R-FC group in the TP53 mutated subgroup (FC 12.1 / R-FC 15.4 months; HR 0.53 (0.31-0.9) P=0.019). Patients with TP53 mutation showed a median OS of 39.3 months whereas median OS was not reached in all other patients (HR 6.01 (4.08-8.87) P<0.001).

Table. Impact of treatment arm and genetic factors on overall survival in CLL patients treated with FC/R-FC (CLL8 trial).

	p=	lower CI (95%)	upper CI (95%)	HR
R-FC	0.019	.432	.927	0.633
IGHV unmutated	0.035	1.035	2.576	1.632
TP53 Mutation	0.002	1.415	4.905	2.634
17p deletion	0.0002	1.831	6.780	3.523

Multivariate analysis was performed by Cox regression including age, stage, treatment arms, IGHV status, genomic aberrations and TP53 mutation. Regarding PFS (n=567), independent prognostic factors were 17p- (HR: 3.6; P<.001), TP53 mutation (HR: 2.2; P<.001), unmutated IGHV (HR: 1.7, P<.001), age (HR: 1.4; P<.001), and R-FC (HR: 0.52; P<.001). Regarding OS (n=580), 17p- (HR: 3.5; P<.001), TP53 mutation (HR: 2.6: P<.001), unmutated IGHV (HR: 1.6; P=.035), and R-FC (HR: 0.6; P=0.019) were identified as independent factors. The analysis of follow-up samples (n=94; collected a median of 1028 days after the initial sample (range 97-1963)) showed an incidence of TP53 mutation of 26.6% (25 / 94). All 17 cases with TP53 mutation before treatment and available follow-up sample, showed the same mutation. Eight follow-up samples showed a TP53 mutation that was not present initially (10.5%; 8/76 samples tested). In conclusion, 17p deletion and TP53 mutation (independent of 17p deletion) are powerful and independent prognostic markers after 1st line FC and R-FC treatment in CLL. When considering the genetic profile in a multivariate model including these, R-FC improves PFS and OS compared to FC in CLL.

0544

HUMAN LEUKOCYTE ANTIGEN MATCHING AND CONDITIONING IMPACT ON LONG-TERM TRANSPLANT OUTCOME AFTER ALLOGENEIC HSCT FOR CHRONIC LYMPHOCYTIC LEUKEMIA: STUDY FROM THE EBMT REGISTRY

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Background. The outcomes of related and unrelated donor hematopoietic stem cell transplantations (HSCT) are strongly affected by the degree of human leukocyte antigen (HLA) matching between the transplant recipient and the donor or cord blood unit. HLA matching plays an important role in engraftment, incidence and severity of graft-versus-host disease (GVHD) and also in overall survival; although this factor is still not validated yet in many hematological malignancies. Objective: To evaluate the impact of HLA matching and difference in matching degree among transplants from unrelated donors (UD) on different outcomes in chronic lymphocytic leukemia (CLL). *Materials and Methods.* we have analyzed 370 CLL patients who underwent allogeneic hematopoietic stem cell transplantation (HSCT) as reported to the EBMT registry between 1995 and 2007. There were 198 HLA-identical siblings and among transplants from UD, there were 31 well matched in high resolution (WMUD), and 141 mismatched (MM) including 30 matched in low resolution. Regarding conditioning, 266 (72%) were RIC and 104 (28%) standard. *Results.* After multivariate adjustment for possible confounders, there was no difference in overall survival (OS) between HLA-identical siblings [5 years OS=55% (48-64)] and WMUD [5 years OS=59% (41-84)] P=0.82. In contrast, OS was significantly worse for MM [5 years OS=37% (29-48) P=0.005] due to a significant excess of transplant related mortality (TRM). Conditioning intensity (RIC) had no significant impact on OS [HR=1.19 (0.78-1.81) P=0.4], relapse [HR=1.69 (0.83-3.44) P=0.14] and TRM [HR=1.01 (0.62-1.64) P=0.9]. *Conclusions.* Our findings support the use of WMUD as equivalent alternative to HLA-matched sibling donors for allogeneic HSCT in CLL, and justify further exploration of RIC in this disease.

Acute myeloid leukemia - Clinical

0545

OUTCOME OF PATIENTS WITH NEWLY DIAGNOSED ACUTE PROMYELOCYTIC LEUKEMIA (APL) TREATED WITH THE COMBINATION OF ALL-TRANS RETINOIC ACID (ATRA), ARSENIC TRIOXIDE (ATO), WITH OR WITHOUT GEMTUZUMAB OZOGAMICIN (GO)

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Background. With the introduction of arsenic trioxide (ATO) as an effective treatment for relapsed acute promyelocytic leukemia (APL), its potential role earlier in the course of treatment is increasingly under investigation. *Aims.* We examined the outcome of patients with newly diagnosed acute promyelocytic leukemia (APL) treated with all-trans-retinoic acid (ATRA) and arsenic trioxide (ATO) with or without gemtuzumab ozogamicin (GO) but without traditional cytotoxic chemotherapy in induction or consolidation. *Methods and patients*. From July 2002 to December 2009, 92 patients with newly diagnosed APL with performance status (PS) ≤2 were treated with a combination of ATRA plus ATO in two studies. All patients signed an approved informed consent form before participation. The first cohort of 47 patients received ATRA (45 mg/m^2 daily) and ATO (0.15 mg/kg daily beginning on day 10 of ATRA). High-risk patients (White blood cell count [WBC] ≥10×10^9/L) received GO 9 mg/m^2 on the first day of induction. From July 2007, the second cohort of 45 patients received ATRA (45 mg/m^2 daily) and ATO (0.15 mg/kg daily) concomitantly on day one of induction. They also received GO 9 mg/m^2 on day 1, if high risk, and any time during induction if the WBC rose to > 30×10^9/L (and more recently if > 10×10^9/L). Consolidation was with 4 courses of ATO administered daily for 5 days/week for 4 weeks every other month as well as ATRA 2 weeks on/2 weeks off for a total of 28 weeks after CR. Monitoring for PML-RARA fusion gene using reverse transcriptase-polymerase chain reaction (RT-PCR) was conducted after induction and throughout consolidation and follow up. If confirmed RT-PCR positive, GO 9 mg/m^2 would be administered with ATRA and ATO every 4-5 weeks (based on recovery of counts) until negative. The median age for the 92 patients was 46 years (range, 14-81 years). Their median presenting WBC was 2.7×10^9/L (0.4-131.4×10^9/L) and their median platelet count was 36×10^9/L (range, 7-261×10^9/L). Sixty four (70%) had low risk and 28 (30%) high risk disease (based only on the presentation WBC < or ≥ 10.0×10^9/L). *Results.* Overall, 90 patients (98%) achieved complete remission (CR) and 2 died at induction. With a median follow-up of 25 months (range, 3 to 99 months), 83 patients remain alive. The estimated 5-year survival rate is 90% and only 5 of the patients achieving a CR (6%) have relapsed. The median overall, relapse-free and event-free survival has not been reached (Figure 1). Two late deaths (beyond 75 months) were in CR and from unrelated cancers. *Conclusions.* The combination of ATRA and ATO (with or without GO) as initial therapy for APL is highly effective and safe; it can potentially substitute chemotherapy containing regimens in high and low risk patients.

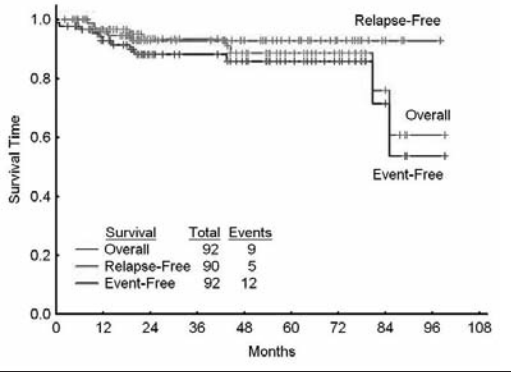

Figure 1. Overall, relapse-free and event-free survival

0546

LONG-TERM OUTCOME OF AML PATIENTS ACCORDING TO THE NEW GENETIC RISK CLASSIFICATION OF THE EUROPEAN LEUKEMIANET RECOMMENDATIONS: EVALUATION OF THE PROPOSED REPORTING SYSTEM IN A COHORT OF 1507 PATIENTS

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Background. Among several prognostic factors in patients with Acute Myeloid Leukemia (AML), cytogenetic aberrations have the strongest impact on prognosis. Whilst there is consensus on classification and prognostic value of favorable risk (FR) and adverse risk (AR) aberrations, most patients display neither favorable nor adverse genetic features, therefore creating a large and heterogeneous group of intermediate risk (IR). Based on literature review and expert consensus, the authors of the recently published European LeukemiaNet (ELN) recommendations on diagnosis and management of AML proposed a subdivision of the IR group into an Intermediate-I group (normal karyotype with absent NPM+/FLT3-ITD- and absent CEBPA+) and a less favorable Intermediate-II group. *Aims.* To assess the prognostic value of the new ELN reporting system for correlation between cytogenetic and molecular genetic data, and clinical data, in a large cohort of patients. *Methods*. Complete data for classification were available for 1507 of 1916 patients treated in the AML96 trial between 1996 and 2004. Patients were assigned to the proposed genetic groups from the ELN recommendations and survival analyses were performed using the Kaplan-Meier method and log-rank tests for significance testing. *Results.* Assignment to risk groups resulted in roughly equal patient numbers in all four genetic groups with 21-29% of all patients per group. The median age of all patients was 59 years (range, 15-87). After a median follow-up time of 7.9 years, significant differences in the probability of relapse (PR) were observed between all groups. The median time to relapse (TTR) in the IR-I, IR-II and AR groups was 1.1, 2.1 and 0.6 years, respectively (p=0.012). The median TTR for the FR group was not reached. Similar effects were observed for disease-free survival (DFS). Separate analyses in the age groups ≤60 years and >60 years revealed that the described differences between IR-I and IR-II were caused by marked effects in the young age group while in elderly patients no difference between IR-I and IR-II were observed. The median overall survival (OS) in all patients was 1.0 year. The median OS was significantly different between the FR group (3.3 years) and the AR group (0.6 years), while there was no difference in OS between the two IR groups (1.1 years each). *Summary/Conclusions.* In our cohort, the ELN classification resulted in significant differences between all four risk groups in terms of PR, TTP and DFS, particularly in younger AML patients. Interestingly, patients in IR-I have a higher risk of relapse than in IR-II, which is most likely due to the presence of FLT3-ITD+ in IR-I. The ELN classification therefore seems to mirror variations in AML biology. Noteworthy, no differences were noted between the IR-I and IR-II groups regarding OS. This may be due to the risk-adjusted treatment approaches applied in the AML96 trial, in which autologous and allogeneic transplantation were applied whenever possible in the IR group. Additional prognostic factors and on-treatment evaluations are desirable for the prediction of OS in the group without favorable or adverse genetic features and for elderly AML patients.

0547

FACTORS DETERMINING CLINICAL RESPONSE TO 5'-AZACITDINE AND SODIUM VALPROATE COMBINATION THERAPY IN PATIENTS WITH HIGH RISK ACUTE MYELOID LEUKAEMIA AND MYELODYSPLASIA

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Background. Demethylating agents and histone deacetylase inhibitors represent important new treatment options in high risk acute myeloid leukaemia (AML) and myelodysplasia (MDS). However the optimal combination of these agents remains to be determined. *Aims.* We therefore evaluated the clinical activity of a novel combination of the demethylating agent 5'-azacitidine (AZA) and the histone deacetylase inhibitor sodium valproate (VAL) with the aim of identifying factors predicting clinical outcome. *Methods.* 71 patients with high risk AML or MDS received treatment with AZA (75 mg/m^2 x 7 days per 28 day cycle) and continuously administered VAL (1-2.5 g daily as tolerated). Patients also received continuous all trans retinoic acid and theophylline. All patients gave informed consent and the clinical trial was approved by the Research Ethics Committee. Clinical responses were assessed using Cheson criteria. Patients achieving a complete remission (CR) or partial response (PR (defined as >50% reduction in bone marrow blasts)) after six cycles continued trial therapies until loss of response. 59 patients commencing treatment had AML (43 relapsed/ refractory disease, 16 untreated) and 12 high risk MDS by IPSS criteria. The median patient age was 66 years (range 32-85). Cytogenetic data was available for all patients: 76% patients were standard, 24% poor and 1% good risk according to MRC criteria. *Results.* A total of 47 patients received 1 or more cycles of AZA. The most common Grade 3-4 non-haematological toxicities were febrile episodes (44%), fatigue (31%) and diarrhoea (13%). Major clinical responses (CR or PR) were observed in 27 (38%) patients. 13 (18%) patients achieved CR or CR with incomplete count recovery (CRi) and 14 (20%) a PR. The median time to achievement of maximal response was 3 cycles. Response duration ranged from 29-799 days. The 1 year overall survival (OS) was 59% (95% CI: 42-73%). The liklihood of achieving a major clinical response (CR or PR) was higher in patients with previously untreated disease in univariate and multivariate analysis. Improved OS was associated with a lower percentage of blasts at the commencement of therapy and the presence of previously untreated disease in univariate analysis. Age, cytogenetic classification and performance status did not impact on the liklihood of achieving a major clinical response or OS. *Conclusions.* These data demonstrate significant clinical activity of AZA and continuously administered VAL in elderly patients with high risk AML and MDS. Importantly clinical activity was not influenced by cytogenetic subgroup or patient age. The liklihood of achieving a major clinical response was higher in patients with no history of prior exposure to chemotherapy emphasising the importance of early deployment of these agents.

0548

LOW DOSE GEMTUZUMAB OZOGAMICIN PLUS FLUDARABINE, CYTARABINE, IDARUBICIN (GO-FLAI) AS INDUCTION THERAPY IN CD33-POSITIVE ACUTE MYELOID LEUKEMIA (AML) PATIENTS YOUNGER THAN 65 YEARS. INTERIM RESULTS FROM A PHASE III MULTICENTER PROSPECTIVE CLINICAL TRIAL (MYFLAI07-NCT.00909168).

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Introduction. We report the interim results from a phase III multicenter clinical trial combining low dose of Gemtuzumab-ozogamicin (GO) with FLAI regimen (Fludarabine, Cytarabine, Idarubicin) as Induction chemotherapy in AML patients younger than 65 years (eudract: 2007-005248-26; ClinicalTrials.gov NCT00909168). *Patients and Methods.* Primary endpoints: Feasibility, Efficacy (CR, PR rate) and Toxicity of GO-FLAI; overall survival (OS) and disease free survival (DFS). Secondary endpoints: Evaluation of Minimal Residual Disease by WT1 (and other biologic markers) expression and monitoring. Feasibility and outcome of consolidation with BMT. One hundred and eighteen consecutive AML patients were included. All patients were younger than 65 with a median age of 51 years (range, 18-65) and CD33 expression exceeded 20% in all cases. The M/F ratio was 58/60, and 80/118 (68%) of patients were poor-risk at diagnosis. The induction regimen (GO-FLAI) included fludarabine (30 mg/sqm) and Ara-C (2 g/sqm) on days 1-5, idarubicin (10 mg/sqm) on days 1, 3, and 5 and GO (3 mg/sqm) on day 6. Hematopoietic stem cell transplant (HSCT) was planned for all high risk AML patients in first complete remission (CR) after consolidation with intermediate doses of Ara-C and idarubicin (ID-AC and IDA). Cytogenetic, multidrug-resistance phenotype, FLT3 and NPM mutation status, WT1 quantitative expression analyses, were performed at diagnosis in all patients. Quantitative WT1 gene expression (with RQ-PCR technique validated by Leukemia Net), cytogenetic (in positive cases) and specific molecular marker analyses were performed after induction to detect and follow Minimal Residual Disease. *Results.* Patients were evaluated for response rate, treatment-related adverse events, OS and DFS. After induction with GO-FLAI, CR rate was 81% (95 of 117 evaluable pts); four patients achieved partial remission (PR) and 15/117 were resistant (Overall response rate was 85%). There were only 3 cases of death during induction (DDI 3%). The haematological and extra haematological toxicity of GO-FLAI was manageable and will be reported in detail; 55% of patients experienced transient and reversible GO infusion-related adverse events (especially fever and chills), but no cases of veno-occlusive disease occurred during chemotherapy or after allogeneic SCT. In the setting of patients who achieved a cytological CR after GO-FLAI, the mean of WT1 copies dropped from 8178±10040/104ABL (at diagnosis) to 185±225 copies/104ABL after induction therapy [P<0.05]. After a median follow-up of 16 months (range 1-50), 88/118 (75%) patients are alive (83/88 in CR). The probability of 2-year OS and DFS were 74% and 70%. Allogeneic and Autologus SCT was performed in 65 (55%) and 19 (16%) patients, respectively. *Conclusions.* The interim results of this trial confirm that GO-FLAI is an effective and well tolerated induction regimen for CD33 positive AML patients younger than 65 years, with a high complete response rate (81%), good disease debulking, favourable safety profile and low DDI (3%), allowing consolidation therapy with SCT early and in a high proportion of cases (84/118, 71%).

0549

A PHASE II MULTICENTRE STUDY WITH ELACYTARABINE IN LATE STAGE ACUTE MYELOID LEUKAEMIA

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Background. Elacytarabine (CP-4055; 5'-O-(trans-9"-octadecenoyl)-1-β-D-arabinofuranosyl cytosine) is a novel cytotoxic nucleoside analogue with similar mechanisms of action to cytarabine, but unlike cytarabine it is independent of nucleoside transporters (e.g. human Equilibrative Nucleoside Transporter 1 (hENT1)) for cellular uptake. *Aims.* To assess the efficacy and safety of elacytarabine monotherapy when given to patients (pts) with late stage acute myeloid leukemia (AML). *Methods.* An open, multicenter study of elacytarabine, enrolling consenting adult pts with PS 0-2, who had received 2 previous chemotherapy regimens and who had refractory/relapsed AML. CR after first salvage therapy lasted < 6 months. Study drug was administered at 2,000 mg/m^2 as a continuous IV infusion (CIV) d1-5 q3w. CR+CRp and overall survival were compared with a historical material of AML patients receiving second salvage therapy (Giles et al, Cancer 2005;104: 547-54) . The population was matched for 6 adverse prognostic factors (adv fact), identified in the historical material, and put into 4 risk groups based on the number of adv fact; low risk group, 1-2 adv fact; intermediate 1: 3 adv fact , intermediate 2: 4 adv fact, high risk group: 5-6 adv fact. An Independent Data Monitoring Committee (IDMC) evaluated the results for futility and toxicity after every 20-pt cohort. Pts were followed for relapse and survival for at least 6 months. Toxicity was graded according to NCI CTCAE v3.0. *Results.* Sixty-one pts (40 male and 21 female) with late stage AML and a median age of 58 years (range 25-82) were enrolled between April'08 and March'09 and received at least one course of elacytarabine. Fifty-three pts had PS 0-1 and 8 had PS 2. The adverse prognostic factors included: duration of CR1< 12 months (43 pts), duration of CR2< 6 months (61), second salvage therapy not including SCT (61), non-inversion16 AML (50), platelet count <50×10^9/L (40), WBC>50×10^9/L (10). Two pts were in the low risk group, 9 pts in the intermediate 1, 20 pts in the intermediate 2 and 30 pts were in the high risk group. Twelve pts had received previous stem cell transplantation (SCT). Nine pts attained CR/CRp, 5 CR and 4 CRp, representing a remission rate of 14.8% vs 2.5% in the historical control (P< 0.0001). The median overall survival was 5.3 months (160 days; range 8 - 411+ days), 3 times that of the historical control of 1.5 months. The 6 month survival rate was 44%. Ten pts were referred to SCT post treatment, including, but not limited to pts in CR. Side effects were predictable and manageable. The most frequently reported related adverse events grade ≥ 3 (CTCAE v3.0) were thrombocytopenia, leucopenia, neutropenia, febrile neutropenia, anemia and lymphopenia. The 30 day all cause mortality following treatment was only 13% vs. 25% in the historical control. *Conclusions.* In this study, elacytarabine showed promising activity, remission rate and overall survival, and manageable side effects in late stage AML patients. A randomized phase III study is planned to further elucidate the effect of elacytarabine in relapsed AML.

Acute lymphoblastic leukemia - Biology

0550

CLONAL GENETIC EVOLUTION IN HUMAN ACUTE LYMPHOBLASTIC LEUKEMIA TRANSPLANTED INTO IMMUNE-DEFICIENT MICE

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Background. Cancer stem cells are believed to represent a distinct subpopulation of tumor cells with self-renewal capacity. The concept of cancer stem cells has come from pioneering studies on acute myeloid leukemias which have defined a subset of cells, named the NOD-SCID-leukemia initiating cells (LIC), characterized by their ability to initiate the disease when transplanted into immune-deficient mice. The cellular characteristics of cancer stem cells are thought to be due to an epigenetic state reminiscent of that of normal stem cells. These characteristics confer a particular resistance to drugs, implying that cancer stem cells are involved in the persistence of tumor cells during treatment and subsequent relapse. *Aims.* Genetic heterogeneity and clonal evolution is another aspect of cancer biology. As it has been shown to occur frequently in acute lymphoblastic leukemia, we hypothesized that it might contribute to leukemia initiation in immune-deficient mice. We aimed to test this hypothesis by comparing the genetic alterations of human primary leukemias and corresponding xenografted leukemias. *Methods* A series of 18 human T-cell acute lymphoblastic leukemias (T-ALL) was successfully engrafted into NOD-SCID or NOD-SCID-IL2Rγ-/- mice. High-density array-CGH was used to compare the pattern of copy number alterations (CNAs) of paired diagnosis and engrafted T-ALL samples. Several CNAs were precisely mapped to sequence the breakpoint junctions and perform specific backtracking by Q-PCR. Re-sequencing of PTEN, WT1, NOTCH1 and FBXW7 genes was performed. *Results.* In 10 out of 18 cases, we found at least one new CNA in engrafted leukemic samples compared to the corresponding diagnostic sample. Strikingly, these CNAs frequently targeted well-known oncogenes or tumor suppressor genes, such as PTEN (n=2 cases), MYC, CDKN2A/p16/ARF, WT1 and LEU2. Moreover, additional mutations in PTEN (n=3) and FBXW7 (n=2) as well as different profiles of NOTCH1 mutations (n=4) were identified between diagnostic and engrafted samples. Backtracking these genomic abnormalities in diagnostic samples showed that they were already present but in a minor subclone, ranging between 0.01 to 6% of the leukemic cells. In some cases, analysis of CNAs profiles demonstrated that the engrafted leukemic clone did not directly derive from the major clone present in the diagnostic sample, but from a common ancestral clone. *Conclusions* These results demonstrate a clonal genetic evolution during leukemia re-initiation from leukemic cells transplanted into immune-deficient mice. The frequent targeting of cancer genes, including genes involved in self-renewal by genomic abnormalities identified in engrafted cells, and their pre-existence in minor leukemic sub-clones at diagnosis suggest that a selection process has occurred. That implies that subclonal oncogenic alterations may participate in the LIC phenotype. These findings have important implications for the cancer stem cell biology.

0551

NEXT GENERATION TRANSCRIPTOME RESEQUENCING IDENTIFIES NOVEL POINT MUTATIONS, GENE EXPRESSION AND ALTERNATIVE SPLICING PROFILES IN BCR-ABL1 POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

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Background. Although the pathogenesis of BCR-ABL1+ ALL is main-

ly related to the expression of BCR-ABL1, additional genetic lesions are supposed to be involved in its development and progression. *Aim.* In order to define the full repertoire of leukemia-related mutations, changes in expression profiles and alternative splicing (AS) events, the leukemia transcriptome of a BCR-ABL1+ ALL patient at diagnosis and relapse was sequenced using a Whole Transcriptome Sequencing (RNA-Seq) approach. The selected cases had previously been profiled by high-resolution SNP and gene expression arrays and candidate gene resequencing. *Methods.* Poly(A) RNA from blast cells was used to prepare cDNA libraries for Illumina/Solexa Genome Analyzer. Obtained sequence reads were mapped to the human genome reference sequence (UCSC hg18) to identify single nucleotide variants (SNVs). Reads that showed no match were mapped to a dataset of all possible splice junctions created in silico to identify AS events. The number of reads corresponding to RNA from known exons was also estimated and a normalized measure of gene expression level (RPKM) was computed. *Results.* RNA-seq analysis generated 13.9 and 15.8 million reads from *de novo* and relapsed ALL samples, most of which successfully mapped to the reference sequence of the human genome. With the exclusion of the T315I mutation in the BCR-ABL kinase domain, seven novel missense mutations were detected after applying stringent criteria to reduce the SNV discovery false positive rate and validating novel substitutions with genomic DNA Sanger sequencing: 4 were exclusively found in the primary ALL sample and affected genes involved in metabolic processes (DPEP1, ZC3H12D, TMEM46) or transport (MVP); 3 missense mutations specific to the relapse sample affected genes involved in cell cycle regulation (CDC2L1) and catalytic activity (CTSZ, CXorf21). Differences in mutational patterns suggest that the leukemia clone from which relapsed cells have been developed was not the predominant one at diagnosis and that relapse specific variants were mutations probably acquired during Ph+ ALL progression. Moreover, 4,334 and 3,651 primary ALL and relapse isoforms with at least one AS event were identified. An average of 1.5 and 1.3 AS per isoform was estimated. The well-known alternatively spliced IKZF1 gene was also detected. Finally, a detailed gene expression profile was obtained indicating that more than 60% of annotated human genes were transcribed in leukemia cells in both diagnosis and relapse phases. Approximately 23% of genes were up-regulated at relapse with respect to diagnosis. Many of these genes affect cell cycle progression (AURORA A, SURVIVIN, PLK1, CDK1, Cyclin A, Cyclin B), suggesting that the loss of cell cycle control and the subsequent increased proliferation play a role in disease progression. Conversely, only 9% of active genes in both samples were down-regulated at relapse with respect to diagnosis. *Conclusions.* Discovery of novel missense mutations, as well as exhaustive alternative splicing and gene expression profiles were achieved for the first time for a BCR-ABL1+ positive ALL demonstrating that RNA-Seq is a suitable approach for identifying a wide spectrum of genetic alterations. Supported by AIL, AIRC, FIRB 2006, European LeukemiaNet, GIMEMA ONLUS.

0552

MIRNA-155 TARGETS BCL6 AND HDAC4 IN A MURINE B-CELL LEUKEMIA MODEL: A PARADIGM SHIFT IN THE ONCOGENIC MECHANISMS OF MICRORNAS

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Background. MicroRNAs (miRNAs), are non-coding RNAs with key regulatory roles in hematopoiesis. Earlier studies showed that miR-155 is over-expressed in several lymphoid and myeloid malignancies. Over-expressing miR-155 in mouse B cells has been shown to induce pre-B cell proliferation followed by high-grade B-cell lymphoma. It is thought that miR-155 exerts its oncogenic activity by targeting directly or indirectly tumor suppressor genes, such as SHIP-1. Despite these advances, further research is needed to uncover the molecular mechanism responsible for miR-155 induced leukemogenesis. *Aims.* Here, we attempt to further characterize the mechanisms by which miR-155 contributes to leukemogenesis by performing genomic wide mRNA gene expression analysis in B cells from the transgenic (TG) miR-155 mice. *Methods.* B cells were isolated from spleens of TG and wild type (WT) mice using MACS B cell isolation kit. Total RNA isolated with Trizol was hybridized into the Affymetrix microarray Chip (mouse 4302 Arrays). Data were normalized using GC-RMA (BRB Array tools). Univariate analysis (Class comparison) was performed to compare TG (n=4) Vs. WT (n=4) samples. Luciferase assay, western blots and qRT-PCR were used to validate miRNA targets. *Results.* We identified 544 genes (229 up and 315 down) differentially expressed in the TG with respect to the WT samples ($P<0.01$, $FDR<0.01$). Interestingly, we observed that the oncogene B cell leukemia 6 (BCL-6) was down regulated two-fold in the miR-155 TG B cells. These results were validated by RT-PCR and immunoblotting. To investigate whether miR-155 targets directly BCL-6, we examined its 3' untranslated region (UTR) for miR-155 binding sites. Although there were two possible miR-155 binding sites in BCL-6 3' UTR, there was no interaction between the BCL-6 3'UTR luciferase reporter and miR-155. Since BCL-6 has been shown to be regulated by acetylation, we reasoned that miR-155 was regulating BCL-6 by increasing BCL6 acetylation and thus degradation by the proteosome. Indeed, immunoprecipitated Bcl6 from total splenocytes of TG mice had increased levels of acetylated versus total Bcl6 as compared to WT control mice. We further identified that histone deacetylase 4 (HDAC4), mRNA and protein expression levels were lower in the miR-155 TG B cells (50% reduction, respectively) and that HDAC4 is a direct target of miR-155 (luciferase assays). Since HDAC4 de-acetylate non histone proteins as well, we reasoned that miR-155-dependent HDAC4 repression resulted in increased BCL-6 acetylation and degradation. Finally, microarray expression of BCL6 targets in the TG samples revealed that three known BCL-6 targets (Chemokine Ligand 3/CCL3, Cyclin D1/ccnd1 and Inhibitor of differentiation/Id2) were significantly up-regulated (6-fold, 3-fold, and 4-fold, respectively) in TG samples with respect to WT. *Conclusions.* Our results indicate that an oncogenic miRNA, like miR-155 targets surprisingly two oncogenes; BCL-6 and HDAC4 and unblock the expression of their targets. This findings enlighten about the complex interplay between tumor suppressor and oncogene pathways affected by a single gene, that when over-expressed results in leukemia. This is a shift in the paradigm of typical oncogenic miRNAs mechanisms, whereby they can act, not only by targeting tumor suppressors but also oncogenes.

0553

LOSS OF THE TUMOR SUPPRESSOR GENE CDKN2A/ARF BY GENOMIC DELETIONS IS A FREQUENT EVENT IN ADULT BCR-ABL1 POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) PATIENTS AND CONTRIBUTES TO DISEASE PROGRESSION

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Background. The chromosome 9p21 locus contains the 40-kb region encoding the p16/CDKN2A (cyclin-dependent kinase inhibitor 2a) tumor suppressor gene and two other related genes, p14/ARF and p15/CDKN2B, all of which encode critical factors for the regulation of cell cycle and/or apoptosis. This locus is a major target in the pathogenesis of a number of human tumors and its inactivation has been also documented in childhood ALL. *Patients and Methods.* In order to assess whether and how it is inactivated in adult BCR-ABL1-positive ALL, we studied 112 adult patients: 78 (70%) were *de novo* ALL, 15 (13%) were unpaired relapsed cases and 19 (17%) were paired relapsed cases. Their median age was 53 years (range: 18-76) and their median blast percentage was 90% (range, 18-99). Affymetrix single nucleotide polymorphism (SNP) arrays (GeneChip® Human Mapping 250K NspI and Genome-Wide Human SNP 6.0) were used to identify at a high resolution copy number changes on 9p21. PCR amplification and mutation screening of all exons by cloning and subsequent sequencing were also performed. *Results.* SNP array analysis revealed CDKN2A/ARF and CDKN2B genomic alterations in 33% and 24% of diagnosed patients, respectively. Deletions were in the majority of cases bi-allelic (73% vs 27%) and had a mean size of 100.8 kb (range, 27 kb-300 kb), ranging from 21.82 Mb to 22.12 Mb. In 70% of cases, deletions were limited to CDKN2A/CDKN2B genes, whereas in 30% they also affected neighbour genes and/or the entire chromosome 9. FISH analysis was performed using three different BAC clones, but since they overlooked microdeletions we only appreciated a mild fluorescent signal reduction. In order to assess whether CDKN2A loss is responsible for progression, 34 patients were analyzed at the time of relapse and a significant increase in the detection rate of CDKN2A/ARF loss (53%) compared to diagnosis ($P=0.04$) was found. In contrast, CDKN2B deletions were found to be not significantly different between diagnosis and relapse (41% vs 24%, $P=0.07$). To assess whether deletions affected CDKN2A/ARF transcript levels, we used the Fluidigm Dynamic Array real-time qPCR assay (Flu-

idigm Corporation, South San Francisco, CA) which enables to perform TaqMan nano-reactions at high sensitivity. This analysis showed that deletions in the 9p21 locus led to a strong down-regulation at the transcript level of CDKN2A/ARF (P=0.0005). Finally, the mutation screening of all exons showed that the 9p21 locus is rarely affected by point mutations, since we only identified the D146N and the R128 in the exon 2 of CDKN2A/ARF and the P83 silent mutation in the exon 2 of CDKN2B gene. These mutations were mutually exclusive and were found in only single cases. *Conclusions.* Loss of the tumor suppressor gene CDKN2A/ARF by genomic deletions is a frequent event in adult Ph+ ALL and it is involved in disease progression. Supported by: European LeukemiaNet, AIL, AIRC, Fondazione Del Monte di Bologna e Ravenna, FIRB 2006, Ateneo RFO grants, Project of integreted program (PIO), Programma di Ricerca Regione - Università 2007 - 2009.

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PARALOG 4 GENES HOXA4 AND HOXB4 INDUCE A STRONG EXPANSION OF B-CELL PROGENITORS IN VITRO IN THE CONTEXT OF E2A-PBX

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The oncogene E2A-PBX1 is the result of a translocation fusing the N-terminal of E2A, containing two activation domains, to the C-terminal of PBX1, a co-factor of the clustered homeobox (HOX) gene proteins. The homeotic cooperative motif (HCM) of PBX is still present in the fusion protein, and biochemical studies have shown that HOX proteins can indeed bind to E2A-PBX1. Moreover, structure function studies demonstrated that the HOX binding motif fused to the E2A activation domains is sufficient to induce cellular transformation. It has thus been suggested that deregulation of the HOX-PBX transcriptional network contributes to the oncogenic properties of E2A-PBX. Previously, we have generated a transgenic mouse model for pre-B ALL induced by E2A-PBX1a and identified HoxA cluster genes as candidate collaborators to E2A-PBX1 in leukemia induction using a proviral insertional mutagenesis screen. Retroviral insertions in the HoxA locus caused the expression of the majority of the HoxA cluster genes (Hoxa3-Hoxa10). Furthermore, we showed that a Hox gene, Hoxb4, genetically interacts with E2A-PBX1 in the induction of leukemia in a murine acute T cell leukemia model. To further investigate the role of Hox genes in E2A-PBX induced B-cell leukemia the expression of HoxA genes was first analysed in E2A-PBX1 primary mouse B-cell leukemias. Except for Hoxa13 the expression of HoxA genes was 100-800 folds higher in B-ALL samples derived from E2A-PBX1/CD3-/- transgenic mice than those derived from MMLV injected mice. In particular the expression of Hoxa4 and -a11 was very high reaching levels of >10000 copies per 25ng RNA. The expression of HoxA genes in B-cells (B220+) isolated from healthy 3-4 months old E2A-PBX1 transgenic mice was comparable to levels measured in control littermates. These data suggest that the activation of HoxA genes is critical in the oncogenic transformation by E2A-PBX1. Hoxb4 and Hoxa4 (unpublished) can expand very early myeloid progenitors and hematopoietic stem cell (HSC). However, it is less clear whether these Hox genes have such a direct effect on B-cell progenitors. To study whether Hox genes expand B-cell progenitors in the context of E2A-PBX1, B-cell cultures were initiated with sorted B220 cells derived from transgenic and control mice and cells were transduced with retroviral vectors for Hoxa4-GFP, Hoxb4-GFP or control-GFP after one week. E2A-PBX1 pro-B cell cultures expressing Hoxa4 and -b4 had grown slightly four days post-transduction (1.2 x and 1.1x, respectively, while cell numbers had decreased in the culture transduced with the control vector (2.7x). B-CFC assays showed that the growth of the E2A-PBX1 B-cell cultures in the presence of Hoxa4 or Hoxb4 was supported by a 4-fold and 3.3-fold expansion of B-cell progenitors. Interestingly, Hoxb4 overexpression in wild type B-cell cultures could not reverse the decline of the B-cell cultures (Hoxa4 not done). Thus the potent expansion of B-CFC by Hoxa4 and Hoxb4 in the presence of E2A-PBX1 only strongly suggests that these genes collaborate with E2A-PBX1 in B-cell leukemogenesis. Currently, we are investigating whether Hoxa4 or -b4 can accelerate B-ALL caused by E2A-PBX1.

Indolent non-Hodgkin's lymphoma

0555

MICRORNA-DEPENDENT MODULATION OF HISTONE ACETYLATION IN WALDENSTROM'S MACROGLOBULINEMIA

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Introduction. Epigenetic regulation of gene-expression, including histone-acetylation, is commonly deregulated in many malignancies leading to aberrant transcription, but microRNA (miRNA)-dependent modulation of histone-acetylation in Waldenstrom's Macroglobulinemia (WM) has not been evaluated yet. *Aims.* To evaluate the histone acetylation status in primary WM cells. To evaluate the role of miRNA9* and miRNA-206 in regulating histone acetylation in WM. 3) To delineate the functional role of miRNA-dependent inhibition of HDAC activity in WM. *Methods.* miRNA- and gene-expression-profiling have been performed on bone-marrow-derived-CD19$^+$ WM cells, compared to their normal cellular counterparts. Data were validated by stem-loop-qRT-PCR. Functional studies were performed on precursor-miRNA-9* and anti-miRNA-206-transfected-WM cells. Effect on signaling cascades have been evaluated by western-blot and immunofluorescence. DNA-proliferation/cytotoxicity/cell cycle/apoptosis were assessed by thymidine incorporation/MTT/PI/Apo2.7 staining, respectively. *Results.* WM cells present with a miRNA signature characterized by increased expression of miRNA-206 and decreased expression of miRNA-9* (ANOVA;P< 0.01). Predicted targets for miRNA-206 and -9* included histone-deacetylases (HDAC4;HDAC5) and -acetyltransferases (Myst3). We first demonstrated that primary WM cells are characterized by unbalanced expression of HDACs and HATs at gene level, responsible for decreased acetylated-histone-H3 and -H4, at protein level and increased HDAC activity. miRNA-206 and -9* played a functional role in regulating histone-acetylation and HDAC activity in WM cells, based on their ability to target HDACs and HATs; leading to induction of toxicity in precursor-miRNA-9*- or anti-miRNA-206-transfected cells, as shown by reduced proliferation rate, cell cycle arrest, induction of apoptosis, supported by PARP-, caspase-8-, caspase-9-cleavage. In addition, miRNA-9* induced autophagy in WM cells by modulating Rab7 and LC3B. *Conclusion*. These findings confirm that histone-modifying genes and HDAC activity are de-regulated in WM cells, partially driven by aberrant expression of miRNA-206 and -9* in the tumor clone; and provide the basis for miRNA-based-targeted therapies in WM.

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PRIMARY WALDESNTROM MACROGLOBULINEMIA (WM) CELLS HARBOR CONSTITUTIVE ACTIVATION OF PI3K/AKT AND MTOR PATHWAYS

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Background. The PI3K/Akt and mTOR pathways play a pivotal role in initiation and progression of malignancies. Therefore, it is critical to examine therapeutic agents that explicitly target these pathways, in tumors that harbor activation of PI3K/Akt/mTOR pathway, such as WM. *Aims.* To evaluate expression level of PI3K/Akt and mTOR in primary WM cells. To evaluate the anti-tumor activity of dual PI3K/Akt and mTOR inhibitor in WM cells in the context of bone marrow milieu. *Methods.* Primary-CD19$^+$ bone-marrow(BM)-derived WM cells; BM stromal cells; WM and IgM secreting low-grade lymphoma cell lines; primary normal CD19$^+$ peripheral-blood-derived (CD19$^+$PB) cells were used. Gene-expression and microRNA-profiling have been performed. Cytotoxicity/DNA synthesis/cell cycle/apoptosis were measured by thymidine uptake/MTT/PI staining/Apo2.7 and flow cytometry analysis, respectively. Cell signaling and apoptotic pathways were delineated by Western Blot and immunofluorescence. *in vivo* homing has been assessed by *in vivo* flow cytometry. *Results.* Primary BM-derived-WM cells present with lower expression of PTEN; higher expression of pospho(p)-Akt, p-mTOR, rictor, raptor, as compared to normal CD19$^+$PB cells. Moreover, microRNA-542-3p and -494 are highly expressed in primary WM cells as compared to normal CD19$^+$PB cells (P<.01); and they both target PTEN, suggesting their role in inhibiting PTEN expression. We tested the dual PI3K/Akt and mTOR inhibitor NVP-BEZ235, which induced cytotoxicity and inhibited DNA synthesis in primary WM cells,

and in IgM-secreting-cell-lines; without cytotoxicity on CD19+PB cells. NVP-BEZ235 inhibited p-Akt/p-mTOR; and downstream-Akt-targeted-proteins (GSK3a/b;p-S6R;p-p70S6). NVP-BEZ235 also inhibited Akt and mTOR *in vitro* kinase activities; as well as rictor and raptor, thus abrogating the rictor-induced-Akt-phosphorylation in WM cells. NVP-BEZ235 also induced significant cytotoxicity in WM cells through targeting forkhead-box-transcription-factors. Finally, NVP-BEZ235 targeted WM cells in the context of BM microenvironment by inhibiting migration, adhesion *in vitro* and homing *in vivo*. *Conclusion*. These studies therefore show that WM cells harbor activation of the PI3K/Akt/mTOR cascades; and dual targeting of the PI3K/Akt/mTOR pathways represents a promising therapy for WM.

0557

RITUXIMAB MAINTENANCE FOR 2-YEARS SIGNIFICANTLY IMPROVES THE OUTCOME OF PATIENTS WITH UNTREATED HIGH TUMOR BURDEN FOLLICULAR LYMPHOMA AFTER RESPONSE TO IMMUNOCHEMOTHERAPY: RESULTS OF THE PRIMA STUDY

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Background and Aims. The GELA sponsored intergroup PRIMA Phase III study was designed to investigate 2-years of rituximab (R) maintenance in follicular lymphoma (FL) patients responding to first line immunochemotherapy, consisting of either 8 cycles of R-CVP, or 6 cycles of R-CHOP or R-FCM (plus 2 additional rituximab infusions) according to each participating centre's pre-specified choice. Patient eligibility criteria included: untreated FL (except grade 3B) in adults requiring treatment for high tumor burden (as defined in Salles et al, Blood 2008). *Methods.* From December 2004 until April 2007, 1217 patients from 25 countries (223 centres) consented to participate in the study. Complete data were available for 1193 patients with the following pre-induction characteristics: median age 56 years [range 22-87]; 52% male; 90% Ann Arbor stage III-IV; 56% bone marrow involvement; 4% ECOG performance status >1; 33% B symptoms; 34% elevated LDH; 32% β2-microglobulin >3mg/L; FLIPI score 0-1 (21%), FLIPI 2 (36%), FLIPI 3-5 (43%). Patients received induction immunochemotherapy with R-CHOP (75%), R-CVP (22%) or R-FCM (3%). Response status at the time of randomization was CR=39%; CRu=32% and PR=28% (others 1%). 1018 eligible patients responding to induction therapy were randomized (stratified by regimen and response to induction) to observation or R-maintenance, 375 mg/m^2 i.v. every 8 weeks for 2 years. *Results.* At the planned interim analysis (ITT: 513 observation, 505 rituximab maintenance), after a median follow-up of 25 months from randomization, a significant (stratified log-rank, P<.0001) improvement in PFS (the primary endpoint) was observed in the rituximab maintenance arm (2-year PFS= 82%; 95%CI [78-86%] compared to 66% [61-70%] in the observation arm; hazard ratio (HR)=0.50; 95%CI [0.39-0.64]). An independent review of response and progression validated the significant benefit observed in the R-maintenance arm. Prespecified exploratory analyses confirmed the overall significant PFS benefit among sub-groups for age (<60 and >60), FLIPI score and response after induction. Time to next anti-lymphoma treatment (HR=0.58 [0.44-0.77]) and time to next chemotherapy (HR=0.60 [0.44-0.81]) were also significantly improved. Response status at the end of maintenance or observation was better in the R-maintenance arm (CR/CRu 67% versus 48%, respectively). No major differences were observed in quality of life assessments scores (FACT-G and EORTC QLQ-C30 scales) between the two arms. At the time of data cut-off, only 34 patients (3.3%) had died and longer follow-up is required to evaluate the effects of rituximab maintenance on overall survival in this study. The most common AEs were infections (22% observation, 37% R-maintenance). Grade 3-4 AEs were reported in 16% (observation) and 22% (R-maintenance) of patients (with neutropenia 1% vs. 4%; and infections 1% vs. 4%, respectively). *Conclusions.* The PRIMA study demonstrates that 2 years of rituximab maintenance therapy after immunochemotherapy as first line treatment for FL results in a significant improvement in patient outcome with acceptable toxicity. In FL patients in need of treatment, 2-year rituximab maintenance after immunochemotherapy should now be considered as a new standard of care.

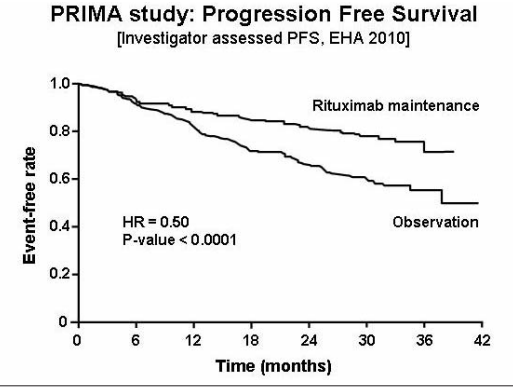

Figure.

0558

PROMISING EFFICACY WITH THE NEW ANTI-CD20 ANTIBODY GA101 IN HEAVILY PRE-TREATED PATIENTS - FIRST RESULTS FROM A PHASE II STUDY IN PATIENTS WITH RELAPSED/REFRACTORY INDOLENT NHL (INHL)

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Background. GA101 is the first fully humanized and glycoengineered type II monoclonal anti-CD20 antibody with Phase I results (NHL/CLL) previously reported (Salles, ASH 2008, 2009; Cartron, EHA 2009; Morschhauser, ASH 2009; Sehn, ASH 2009). *Methods.* 40 patients (all signed informed consent) were randomized to receive GA101 in a low-dose (LD, n=18) or a high dose (HD, n=22) cohort. GA101 was given on d1, d8, d22 and q21 days for total of 9 infusions (6 months). In the LD cohort, GA101 was given 400mg all infusions; in the HD cohort, d1 and d8 at 1600mg and 800mg thereafter. Primary endpoint was response rate, with secondary endpoints of safety and pharmacokinetics. *Results.* Patients (Table 1) were heavily pre-treated (median 4 prior therapies) with 60% of patients not responding to or relapsing within six months after a previous rituximab-containing regimen (rituximab refractory). There were no significant differences in demographics and baseline tumour burden between the two cohorts. 75% of patients completed all scheduled 9 infusions. GA101 was well tolerated in both cohorts with the most common AEs being infusion related reactions (LD 72%, HD 73% of patients), mostly of G1-2. 11 patients experienced at least one SAE, with 5 related to GA101 (LD n=1, HD n=4). During treatment, related G3-4 hematological AEs were transient neutropenia (n=3 in HD) and thrombocytopenia (n=1 in HD), four patients experienced at least one G3-4 Infection (LD n=1, HD n=3). GA101 pharmacokinetics was

similar to rituximab, characterized by two clearance components, one linear and one time-dependent saturable component consistent with target-mediated disposition. 38/40 patients were evaluable for end of treatment response (EOR), evaluated 4 weeks after last infusion, 44 weeks after treatment start. EOR was 17% (3 PR, 6 SD, 7 PD, 2 UNK) in the LD cohort, and 55% (2 CR, 10 PR, 6 SD, 4 PD) in the HD cohort. Of note, 7/24 rituximab-refractory patients (6 HD, 1 LD) responded, with 4 in HD and 1 in LD with an ongoing response. An additional refractory patient in the HD cohort with SD at EOT converted to PR post-treatment. Responses occurred across all FcγIIIR genotypes in both cohorts: of 3 responders in LD, 2 patients with F/F genotype, other unknown; of 12 responders in HD; 5 F/F, 7 F/V. *Conclusion.* In this group of heavily pre-treated iNHL patients, single-agent GA101 was safe with a high response rate in HD cohort (55%), and responses also observed in rituximab-refractory patients (HD 55% [6/11]), supporting a possible dose-response relationship.

	LD Cohort (400mg)	HD Cohort (1600/800mg)	All
n	18	22	40
Sex (M/F)	12/6	13/9	25/15
Follicular histology (n)	14	20	34
Median age (range)	51 (42-79)	61.5 (44-76)	60.5 yrs (42-79)
Median # of prior treatments (range)	5 (1-9)	4 (1-13)	4 (1-13)
Previous rituximab (n)	18	21	39
Rituximab refractory (n)	13	11	24
Prior stem cell transplant (n)	5	5	10

Table 1 Baseline patient characteristics.

0559

CD3/CD19 BISPECIFIC BITE® ANTIBODY BLINATUMOMAB TREATMENT OF NON-HODGKIN LYMPHOMA (NHL) PATIENTS: 60 µG/M²/D BY CONTINUOUS INFUSION IS TOLERABLE AND RESULTS IN DURABLE RESPONSES

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Blinatumomab is a CD19/CD3-bispecific antibody construct of the bispecific T cell engager (BiTE®) class that induces cytotoxic T effector memory cells to kill CD19 expressing malignant B cells. We have previously reported that blinatumomab could be delivered by continuous intravenous (CIV) infusion over 4-8 weeks at doses that depleted peripheral B cells and induced a proliferation of T effector cells. Initial infusion doses of 90 µg/m²/d exceeded the maximal tolerable dose. Here, we update the results of an ongoing phase 1 trial of patients with relapsed indolent NHL treated at a dose of 60 µg/m²/d for 4-8-weeks by CIV infusion with single-agent blinatumomab. In total, 14 patients with mainly follicular or mantle cell subtypes were treated at 60 µg/m²/d during the first treatment cycle. 13/13 evaluable patients showed an objective response (9 PR and 4 CR). As of February 2010, response duration was up to 27+ months. Median follow-up for response duration was 13 months with 8 out of 13 responses ongoing. The single patient that was not evaluable for response experienced a fully reversible, neurological adverse events leading to early discontinuation of treatment. Of the 13 responders, five patients had adverse events leading to discontinuation which were all fully reversible (one port infection and four neurological symptoms). A predictive biomarker, low peripheral blood B:T cell ratio, identified patients with a higher frequency of reversible neurological adverse events. Therefore, we implemented a lower initial dose for 1-2 weeks (5 and/or 15 µg/m²/d, n=5 patients) followed by a maintenance dose of 60 µg/m²/d. A lower starting dose appeared to substantially ameliorate initial adverse events to an extent that treatment could be continued without interruption. Taken together, our data confirm high single-agent activity of 60 µg/m²/d blinatumomab infused for 4-8 weeks with long lasting remissions and a favorable risk/benefit profile. Evaluation of safety and clinical efficacy in other subtypes of NHL and a uniform dose schedule is ongoing.

PRESIDENTIAL SYMPOSIUM

0560

DASATINIB COMPARED TO IMATINIB IN PATIENTS WITH NEWLY DIAGNOSED CHRONIC-PHASE CHRONIC MYELOGENOUS LEUKEMIA (CML-CP): RESULTS FROM THE RANDOMIZED PHASE 3 DASISION TRIA

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Background. Dasatinib is a highly potent BCR-ABL kinase inhibitor. Once daily dasatinib induces rapid, durable complete cytogenetic response (CCyR) in a substantial proportion of patients with imatinib resistance, suboptimal response, or intolerance. Of patients with newly diagnosed CML-CP, a significant proportion fails to achieve an optimal response with imatinib. Lack of CCyR by 12 months is associated with decreased long-term progression-free survival (PFS). *Aims.* The DASISION trial compares the efficacy and safety of dasatinib to that of imatinib as first-line treatment of CML-CP. Results after a minimum follow-up of 12 months are presented. *Methods.* 519 adults with Ph^+ CML-CP stratified by Hasford scores were randomized to receive dasatinib 100 mg once daily (n=259) or imatinib 400 mg once daily (n=260). The primary endpoint was rate of confirmed CCyR (cCCyR; CCyR on 2 consecutive assessments; any # of metaphases) by 12 months. Other endpoints were rates of and times to CCyR and major molecular response (MMR; ≤0.1% BCR-ABL transcripts on the International Scale).

Table. EHA Dasatinib table.

n (%)	Dasatinib	Imatinib	P
CCyR ≥20 metaphases			
3 months	140 (54)	80 (31)	
6 months	189 (73)	154 (59)	
9 months	203 (78)	174 (67)	
12 months	216 (83)	186 (72)	0.0011
MMR			
3 months	21(8)	1 (<1)	
6 months	70 (27)	21(8)	
9 months	101 (39)	48 (18)	
12 months	119 (46)	73 (28)	<0.0001

Results. Baseline patient characteristics were well balanced across arms. Median treatment duration was 14 months for each drug. 85% of patients on dasatinib and 81% on imatinib remained on study; 5% and 4.3% discontinued for adverse drug reactions (ADR), respectively. Median dose intensity was 99 mg/day for dasatinib and 400 mg/day for imatinib. The cCCyR rate was superior for dasatinib vs. imatinib (77% vs. 66%, P=0.0067). Both CCyR and MMR rates by 12 months (Table) were higher for dasatinib vs. imatinib (CCyR: 83% vs. 72%; MMR: 46% vs. 28%). MMR was obtained significantly faster with dasatinib than with imatinib (HR 2.0, P<0.0001), as was CCyR (HR 1.5, P<0.0001). Rates of CCyR and MMR were higher for dasatinib than for imatinib across all Hasford risk groups. Rates of transformation to accelerated phase or blast crisis were 1.9% for dasatinib and 3.5% for imatinib. Grade 3/4 cytopenias were as follows (dasatinib vs. imatinib): anemia (10% vs. 7%), neutropenia (21% vs. 20%) and thrombocytopenia (19% vs. 10%); 4 patients on dasatinib and 3 on imatinib discontinued treatment due to drug-related cytopenias. Thirteen patients (5%) on dasatinib and 12 (5%) on imatinib experienced gastrointestinal or other bleeding. Non-hematologic ADRs (all grades) occurring in ≥10% of patients (dasatinib vs. imatinib) included fluid retention (19% vs. 42%; including pleural effusion, all grade 1 or 2, 10% vs. 0%), nausea (8% vs. 20%), vomiting (5% vs. 10%), myalgia (6% vs. 12%), muscle inflammation (4% vs. 17%) and rash (11% vs. 17%). *Summary/Conclusions.* Dasatinib once daily resulted in significantly higher and faster rates of CCyR and MMR than imatinib. Overall, both treatments were well tolerated. Given the strong predictive value of 12-month CCyR and MMR for long-term PFS, dasatinib as initial therapy may improve long-term outcomes in patients with newly diagnosed CML-CP.

0561

VITAMIN D RECEPTOR IS ESSENTIAL FOR NEURONAL CONTROL OF HEMATOPOIETIC STEM CELL NICHE

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Hematopoietic stem/progenitor cells (HSPCs) are released from the bone marrow (BM) to the circulation by granulocyte-colony stimulating factor (G-CSF) via sympathetic nervous system (SNS)-mediated osteoblast suppression (Katayama *et al*. Cell 2006). Because the orientation of HSPCs in their osteoblastic niche is reported to be guided by $[Ca^{2+}]$, we speculated a co-operation between calcium regulating hormones (CRHs) and SNS to regulate HSPC trafficking. In this study, we found that vitamin D receptor (VDR), the receptor for a major CRH $1,25(OH)_2D_3$, is essential for G-CSF-induced osteoblast suppression via β2-adrenergic signals and for subsequent HSPC mobilization. G-CSF failed to mobilize HPCs (75% reduction assessed by CFU-Cs, n=7, P<0.001) and HSCs (95% reduction assessed by competitive repopulating units at 6 months posttransplant, n=5, P<0.05) in *Vdr*-/- mice. Similar results were obtained from animals grown on high calcium diet in which the major phenotype of VDR deficiency, the rickets type II, was restored. Reciprocal BM transplantation models revealed that this impaired mobilization originated from the defect in microenvironment but not in hematopoietic cells. Despite the highly impaired mobilization, mRNA and protein levels of BM CXCL12 were drastically down-regulated after G-CSF-treatment even in *Vdr*-/- mice, suggesting a possibility that the decrease of CXCL12 level in the BM is not enough or not fundamental for G-CSF-induced mobilization. It is well known that osteocalcin is produced by mature osteoblasts and its level in circulation is decreased during G-CSF-induced mobilization. Plasma osteocalcin levels in steady-state were similar between *Vdr*+/+ and *Vdr*-/- littermates. Following G-CSF-treatment, it was significantly decreased in *Vdr*+/+ mice (29% reduction, n=6, P<0.05) whereas no reduction was observed in Vdr-/- littermates. The number of stretched/spindle shaped (Type III and IV mature) osteoblasts evaluated by histomorphometry was markedly decreased (63% reduction, n=4, P<0.05) in Vdr+/+ mice after G-CSF-treatment whereas it was remained unchanged in Vdr-/- littermates. These data suggest that activity and the number of mature osteoblasts are not decreased by G-CSF in the absence of VDR. Next we assessed the connection between sympathetic stimuli and VDR in mature osteoblasts. Injection of pan-β adrenergic receptor (AR) agonist isoprenaline into normal mice induced rapid increase of Vdr mRNA in the BM in two hours (7.6 fold, n=6, P<0.001). Single dose of G-CSF also induced significant increase of Vdr mRNA in the BM in three hours (1.3 fold, n=4-5, P<0.01). We also evaluated the alterations of VDR at protein and mRNA levels in mature osteoblasts by β-agonists *in vitro*. Isoprenaline-treatment induced great increase of VDR protein and mRNA in MC3T3-E1 and primary calvarial osteoblasts in two hours (mRNA: 7.5-7.8 fold, n=3, P<0.05) and β2-selective agonist clenbuterol displayed similar or more efficient VDR induction, suggesting that the role of VDR in osteoblastic niche is likely inducible by β2-AR signaling. Collectively, our results demonstrate a novel function of active vitamin D3 as a critical mediator of neuronal control of HSPC trafficking and how it contributes to brain-bone-blood integration in an unanticipated way distinct from other classical CRHs.

0562

ZOLEDRONIC ACID (ZOL) PROLONGS TIME TO FIRST SKELETAL-RELATED EVENT (SRE) AND SURVIVAL VERSUS CLODRONATE IN NEWLY DIAGNOSED MULTIPLE MYELOMA (MM): MRC MYELOMA IX TRIAL RESULTS

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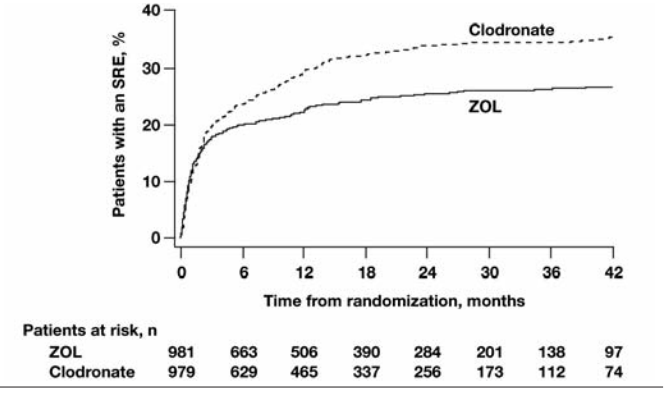
Background. In addition to prolonging survival, an important goal of MM management is the prevention of potentially debilitating sequelae including renal failure and SREs. Bisphosphonates can prevent SREs. However, the comparative SRE-preventing efficacies and renal safety profiles of ZOL and clodronate have not been evaluated in a large head-to-head randomized study in MM. Furthermore, ZOL was reported to improve survival versus no ZOL in a small study in patients with newly diagnosed MM (Aviles *et al.* Med Oncol 2007); therefore, effects on disease outcomes may also be possible. *Aims.* The MRC Myeloma IX study compared primary therapy regimens for patients with newly diagnosed MM. Randomization to ZOL and clodronate was included within each primary-therapy group to allow comparisons between disease outcomes, SREs, safety, and survival between these 2 agents. *Methods.* Newly diagnosed MM patients were randomized to intravenous ZOL (4 mg, dose-adjusted based on renal function) every 3-4 weeks or daily oral clodronate (1600 mg) plus antimyeloma therapy. A diagnosis of myeloma bone disease was not a study requirement; therefore, bisphosphonate use was off-label in some patients. Treatment continued at least until disease progression, and all patients were evaluated for disease outcomes, SREs, and safety before disease progression, and survival after disease progression or discontinuation of study medication. All patients provided written informed consent. Time to first SRE was assessed using the cumulative incidence function, and survival was estimated using the Kaplan-Meier method, adjusting for antimyeloma therapies. A Cox proportional hazards model was used to assess the hazard ratio (HR) and associated 95% confidence interval (CI) for SRE risk adjusting for the effects of antimyeloma therapies, minimization factors, treatment pathways, and prior SRE at baseline. ISRCTN68454111.



Figure

Results. A total of 1960 patients were evaluable, with a median follow-up of 3.7 years. Mean age for ZOL and clodronate groups was 65 years, IgG was the predominant subtype, and approximately 70% of patients in each group had bone disease. Most patients in each group did not experience an SRE. However, after the first 3 months, a smaller proportion of patients at each timepoint developed a first SRE in the ZOL versus clodronate groups (graph). Overall, ZOL significantly reduced the risk of first SREs by 26% versus clodronate in the adjusted Cox model (HR=0.74; 95%CI: 0.62, 0.87; P=.0004). SREs were more prevalent among patients with bone disease at baseline versus those without; ZOL reduced the risk of first SRE in both groups. ZOL significantly increased survival by 5.5 months compared with clodronate (median 50 versus 44.5 months, respectively; P=04). The survival benefit with ZOL remained statistically significant after adjustment for between-group differences in SREs (P=.04). Both bisphosphonates were generally well tolerated. There was no significant between-group difference in the rate of acute renal failure (60 [6.1%] patients with clodronate and 57 [5.8%] patients with ZOL). *Conclusions.* In MRC Myeloma IX, ZOL significantly delayed the onset of SREs and prolonged survival compared with clodronate in patients with newly diagnosed MM receiving antimyeloma therapy. Rates of renal failure were low overall and comparable between groups.

ZOLEDRONIC ACID (ZOL) SIGNIFICANTLY INCREASES PROGRESSION-FREE SURVIVAL (PFS) VERSUS CLODRONATE AND MAY IMPROVE RESPONSE RATES IN NEWLY DIAGNOSED MULTIPLE MYELOMA (MM): MRC MYELOMA IX TRIAL RESULTS

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Background. There is no consensus on optimal treatment for patients with newly diagnosed MM. In addition to standard cytotoxic and antiangiogenesis therapies, bisphosphonates have recently shown anticancer effects beyond their skeletal-related event–preventing indication. Of note, in patients with breast cancer, ZOL reduced the risk of disease recurrence by 36% (P=.01) versus adjuvant endocrine therapy alone (Gnant, *et al*. N Engl J Med 2009). *Aims*. To compare the efficacy of various first-line treatment options for MM and to compare the effects of ZOL and clodronate. *Methods.* The MRC Myeloma IX study assigned newly diagnosed MM patients to either intensive or non-intensive induction therapy pathways based on current guidelines. Intensive pathway patients were randomized to 21-day cycles of either CVAD or CTD. After achieving best response, patients were treated with high-dose melphalan (200 mg/m^2) and stem-cell transplant. Non-intensive pathway patients were randomized to either MP or reduced-dose CTD, each of which was continued until best response. Responses were graded per modified-EGMT criteria. Each of the 4 treatment groups were randomized to intravenous ZOL (4 mg; adjusted for renal function) every 3-4 weeks or daily oral clodronate (1600 mg), which was continued at least until disease progression. After initial therapy, eligible patients were again randomized to either add daily thalidomide maintenance therapy or continue with only bisphosphonate therapy. All patients were evaluated for disease outcomes and safety. PFS was assessed by Kaplan-Meier analyses, and between-group hazard ratios (HR) were based on Cox models stratified by treatment pathway. All patients provided written informed consent. ISRCTN68454111. *Results*. A total of 1960 patients were evaluable, with a median follow-up of 3.7 years. Most patients had ISS stage ≤2, IgG was the predominant subtype, and approximately 70% of patients in each group had bone disease (bisphosphonate therapy was therefore off-label in 30% of patients). Overall, ZOL significantly prolonged median PFS by 2 months versus clodronate (19.5 versus 17.5 months; P<.05). PFS benefits were comparable in each treatment pathway. Complete or very-good-partial responses were achieved by significantly more patients treated with ZOL (85 [20.0%]) versus clodronate (60 [14.2%]) in the non-intensive pathway (P=.03), and a similar proportion of patients treated with ZOL (200 [36.0%]) and clodronate (193 [34.7%]) in the intensive pathway (P=.66). However, in both treatment pathways fewer ZOL- versus clodronate-treated patients developed new osteolytic bone lesions (non-intensive: 5.2% versus 9.9%, respectively; intensive: 4.3% versus 9.5%, respectively). There were also trends toward fewer disease- or treatment-related–deaths within the first 60 days on study (during induction therapy) for ZOL- versus clodronate-treated patients (intensive: 13 [2.3%] versus 18 [3.2%] respectively; non-intensive: 11 [2.6%] versus 23 [5.4%]. respectively). ZOL and clodronate were generally well tolerated, and confirmed osteonecrosis of the jaw was infrequent with both (ZOL, 3.6%; clodronate, 0.3%). *Conclusions*. In addition to its established indication for preventing skeletal-related events, ZOL significantly improved PFS and produced trends toward increased response rates to induction therapy versus clodronate in patients with newly diagnosed MM in MRC Myeloma IX. Therefore, ZOL may have clinically meaningful anticancer effects when combined with chemotherapy.

0563

INACTIVATING MUTATIONS OF THE HISTONE METHYLTRANSFERASE EZH2 ARE ASSOCIATED WITH CHROMOSOME 7 ABNORMALITIES IN MYELOID DISORDERS

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Abnormalities of chromosome 7q are common in myeloid malignancies but no specific target genes have been identified. Recently, we and others reported that 7q acquired uniparental disomy (aUPD) is seen in MDS/MPN and MDS but is uncommon in AML. To identify further cases with 7q aUPD, we performed genomewide single nucleotide polymorphism (SNP) analysis using Affymetrix SNP 6.0 arrays on 148 MDS/MPN cases. Sixty-three tracts of copy number neutral homozygous SNP calls >20 Mb were seen in 46 (31%) of cases, of which 12 involved 7q. The minimally affected region was 52 Mb spanning 7q22.3-7qter. Resequencing 15 genes excluded the involvement of tyrosine kinases and several other candidate genes. To search for microdeletions we designed a targeted high density comparative genomic hybridization array; analysis of 8 cases identified a 400kb deletion at 7q36.1 that encompassed C7orf33, CUL1 and EZH2. Sequencing revealed a frameshift mutation in the residual EZH2 allele of the deleted case, plus homozygous EZH2 mutations in 9/12 cases with 7q aUPD. To determine the prevalence of EZH2 variants, we scanned all exons in 518 cases with MPN, MDS/MPN or MDS. After excluding known polymorphisms, 39 additional heterozygous, hemizygous or homozygous variants were identified in 32 cases. Three patients had -7 on cytogenetic analysis, indicating that both aneuploidy and aUPD can lead to loss of the wild type EZH2 allele. To determine if EZH2 is more generally associated with chromosome 7 loss, we screened AML with -7/7q (n=54) but found no mutations. Of the 49 EZH2 variants, 26 were missense, 21 were predicted to result in premature chain termination mutations and 2 were in frame deletions. The truncating mutations were dispersed throughout the gene whereas the missense mutations mostly targeted evolutionarily highly conserved residues in domain II and the CXC/SET region. Analysis of T-cell DNA showed that 5 of 5 variants analyzed were acquired. Mutations were seen in diverse diseases but were most common in MDS/MPN (27/219; 12%) and primary or secondary myelofibrosis (4/30; 13%). Univariate analysis in MDS/MPN indicated that EZH2 mutations were associated with a poor prognosis compared to cases without mutations (overall survival: 39 months vs. 13 months [P=0.0006]; progression-free survival: 30 months vs. 17 months; [P=0.044]). EZH2 encodes the catalytic subunit of the Polycomb repressive complex 2 (PRC2), the highly conserved histone H3 lysine 27 methyltransferase that influences stem cell renewal by epigenetic repression of genes involved in cell fate decisions. The biallelic truncating mutations we identified were clearly inactivating. To test if this was also the case for missense variants, we infected Sf9 cells with baculoviruses expressing FLAG-tagged EZH2 mutants along with the PRC2 components EED and SUZ12. Following immunoprecipitation and H3K27 methyltransferase assays, all four CXC/SET domain mutants tested were found to abrogate or greatly reduce EZH2 catalytic activity. EZH2 has been reported previously to have oncogenic activity and its overexpression has been causally linked to differentiation blocks in epithelial tumors. Unexpectedly, the mutations we identified abrogated histone methyltransferase activity, suggesting that EZH2 acts as a tumor suppressor in myeloid malignancies.

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ORAL DABIGATRAN VERSUS ENOXAPARIN FOR THROMBOPROPHYLAXIS AFTER PRIMARY TOTAL HIP ARTHROPLASTY: THE RE-NOVATE II RANDOMISED TRIAL

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Background. Extended thromboprophylaxis after hip arthroplasty reduces the risk of venous thromboembolism (VTE). As oral dabigatran offers practical advantages over subcutaneous enoxaparin in the post-discharge setting, we compared the efficacy and safety of these treatments in this indication. *Methods.* In this double-blind, non-inferiority trial, 2055 patients undergoing total hip arthroplasty were randomized to treatment for 28 to 35 days with oral dabigatran, 220 mg once-daily, starting with a half-dose 1 to 4 hours after surgery, or subcutaneous enoxaparin 40 mg once-daily, starting the evening before surgery. The primary efficacy outcome was a composite of total VTE (venographic or symptomatic) and death from all-causes. The main secondary composite outcome was major VTE (proximal deep-vein thrombosis, nonfatal pulmonary embolism) plus VTE-related death. The main safety outcome was major bleeding during treatment. *Results.* The median treatment duration was 32 days. In total, 2013 were treated, of whom 1577 operated patients were included in the primary efficacy analysis. The primary efficacy outcome occurred in 7.7% (61 of 792) of the dabigatran group versus 8.8% (69 of 785) of the enoxaparin group, absolute risk difference -1.1% (95% confidence interval [CI], -3.8 to 1.6%); P<0.0001 for the pre-specified non-inferiority margin. The main secondary efficacy outcome occurred in 2.2% (18 of 805) of the dabigatran group versus 4.2% (33 of 794) of the enoxaparin group (absolute risk difference -1.9%, (95% CI,-3.6% to -0.2%; P=0.03). Major bleeding occurred in 1.4% of the dabigatran group and 0.9% of the enoxaparin group (P=0.40). The incidence of adverse events during treatment did not differ significantly between the groups. *Conclusions.* Extended prophylaxis with oral dabigatran 220 mg once-daily was as effective as subcutaneous enoxaparin 40 mg once-daily in reducing the risk of VTE after total hip arthroplasty. The risk of bleeding and safety profiles were similar.

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DEREGULATION OF C/EBPG RESULTS IN DIFFERENTIATION ARREST IN ACUTE MYELOID LEUKEMIA

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CCAAT/enhancer-binding proteins (C/EBPs) are a family of transcription factors in which 6 core members have been identified: C/EBPA, C/EBPB, C/EBPG, C/EBPD, C/EBPE, and C/EBPZ. These transcription factors play important roles in differentiation and growth control in various tissues. In the hematopoietic system, C/EBPs, with the exception of C/EBPG, have been shown to regulate different stages of granulocytic development, and their aberrant expression has been observed in acute myeloid leukemia (AML). Although C/EBPG function has been partially described in the erythroid and lymphoid lineage, little is known about the role of this transcription factor during myelopoiesis or AML. In the present study, we investigated C/EBPG expression in 547 cases of *de novo* human AML and found that C/EBPG is highly upregulated in a specific subset of AML samples characterized by silencing of C/EBPA. Similarly, in C/EBPA conditional knockout mice, we found that ablation of C/EBPA in the LKS (lineage- c-kit+ Sca-1+) stem/progenitor cells caused an upregulation of C/EBPG. Reintroduction of C/EBPA into C/EBPA knockout LKS by retroviral infection resulted in C/EBPG downregulation, demonstrating a direct relation between C/EBPG expression and absence of C/EBPA. Supporting this hypothesis, DNA promoter microarrays (ChIP on Chip) showed that C/EBPA binds to the C/EBPG promoter, repressing C/EBPG expression. Next, to study the contribution of C/EBPG to the pathogenesis of AML we overexpressed C/EBPG in an *in vitro* differentiation model, and observed that sustained expression of this transcription factor induces a neutrophilic differentiation arrest. Similar neutrophilic arrest has been described in C/EBPA conditional knockout mice, resulting in their lack of mature neutrophils in the bone marrow and blood. Our observations that LKS from this mouse model have high levels of C/EBPG mRNA and that sustained C/EBPG expression blocks granulocytic differentiation led us to hypothesize that downregulation of C/EBPG is required for neutrophilic differentiation in stem/progenitor populations *in vivo*. To test this hypothesis, we designed several shRNA constructs specifically targeting C/EBPG, and examined the effect of knocking down CEBPG in the CEBPA knockout LKS. We observed that downregulation of C/EBPG expression in the C/EBPA deficient LKS was sufficient to completely restore granulocytic differentiation, as shown by *in vitro* cultures and bone marrow transplantation experiments. Altogether, we identify for the first time a role for C/EBPG in AML. Our results indicate that C/EBPG mediates the myeloid differentiation arrest induced by C/EBPA deficiency and suggest that targeting this transcription factor represents an alternative therapeutic strategy for AML.

Myeloma and other monoclonal gammopathies - Clinical 1

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A PHASE 3 STUDY TO DETERMINE THE EFFICACY AND SAFETY OF LENALIDOMIDE COMBINED WITH MELPHALAN AND PREDNISONE IN PATIENTS = 65 YEARS WITH NEWLY DIAGNOSED MULTIPLE MYELOMA (NDMM)

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Background. Lenalidomide is an oral IMiD® immunomodulatory agent with proven efficacy in MM patients. A phase 1/2 trial in elderly patients with NDMM demonstrated that treatment with lenalidomide in combination with melphalan and prednisone (MPR), elicited a high overall response rate (ORR) resulting in prolonged overall (OS) and progression-free survival (PFS) with acceptable toxicity (Palumbo. Clin Lymphoma Myeloma. 2009;9:145-150). *Aims.* A prospective, randomized phase 3 trial compared safety and efficacy of MPR followed by lenalidomide (MPR-R), MPR followed by placebo (MPR), and MP plus placebo followed by placebo (MP) in elderly patients with NDMM. *Methods.* 459 patients aged ≥65 years with NDMM were randomized to receive MPR-R, MPR, or MP. Patients received melphalan 0.18 mg/kg (D1-4), prednisone 2 mg/kg (D1-4), with or without lenalidomide 10 mg/day (D1-21) for nine 28-day cycles. M and R doses were simultaneously reduced for myelosuppression. Following 9 cycles of MPR, patients received continuous treatment with lenalidomide (10 mg/day; D1-21) or placebo until progression; MP patients received placebo until progression. The primary comparison was MPR-R vs MP. Data from a preplanned interim analysis at 50% of events (median follow-up of 9.4 months) are summarized here and the updated results with 70% of events will be presented at the meeting.

Table.

	MPR-R (n = 152)	MPR (n = 153)	MP (n = 154)	*P* Value (MPR-R vs MP)
ORR (CR+VGPR +PR)	77%	67%	49%	< .001
≥ VGPR PR	32% 45%	33% 34%	12% 37%	< .001 ---
Time to first response, months	1.9	1.9	2.8	< .001
PFS, months	Not reached	13.2	13.0	< .001
PR, partial response.				

Results. MPR-R resulted in a higher ORR, higher rates of complete response (CR) and very good partial response (VGPR), more rapid responses, and longer PFS compared with MP (Table). The secondary comparison of the trial (MPR-R vs MPR) demonstrated that continuous therapy with lenalidomide extended PFS compared to placebo (not reached and 13.2 months, respectively; P=.002). A landmark analysis of PFS in patients remaining on therapy following cycle 9 demonstrated that continuous treatment with lenalidomide versus placebo (regardless of the induction arm) resulted in a 75% reduced risk of disease progression (P<.001; HR: 0.245; 95% CI [0.126, 0.476]). MPR-R had a favorable safety profile with minimal cumulative toxicities; only 16% of patients discontinued due to adverse events (AEs). Grade 3/4 neutropenia, neutropenic fever, thrombocytopenia, and anemia occurred in 70%, 7%, 37%, and 23% of patients receiving MPR-R and 29%, 13%, and 17% of patients receiving MP; no grade 3/4 peripheral neuropathy was observed. Importantly, incidence of AEs after cycle 9 was very low, with only 1% patients experiencing grade 3/4 neutropenia and no thrombocytopenia. *Conclusions.* MPR-R elicited higher and more rapid respons-

es resulting in prolonged PFS. Additionally, lenalidomide taken continuously versus a fixed number of cycles significantly extended PFS with incidence of AEs comparable to placebo during the continuous therapy period. These data suggest that continuous use of lenalidomide is superior to regimens of limited duration and provides sustained disease control to patients with NDMM.

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MP VERSUS MPT FOR PREVIOUSLY UNTREATED ELDERLY PATIENTS WITH MULTIPLE MYELOMA: A META ANALYSIS OF SURVIVAL OF 1682 INDIVIDUAL PATIENT DATA FROM 6 RANDOMIZED CLINICAL TRIALS

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Background. Randomized clinical trials including previously untreated patients who were uneligible for high dose treatment were initiated in Italy (GIMEMA 331 pts), France (IFM 321 and 229 pts), Netherlands/Belgium (HOVON 333 pts), Nordic countries (NMSG 357 pts) in 2000-02 and in Turkey (TMSG 114 pts) 2006. These groups in European Myeloma Network have agreed on a common analysis of their individual patient data. *Methods.* An electronic search for publications, abstracts and presentations about MP vs. MPT in multiple myeloma found no trial additional to the 6 presented here. The protocol for the common analyses was finalized after discussions with participants from all groups. All patients from the original studies have been entered into a common data base. Daily dose of thalidomide was 100 mg until relapse (Italy), 200-400 mg in patients below 65 years and 100 mg in patients above 65 years mg in 72 weeks (12 cycles) (France), 400 mg until plateau phase, maintenance 200 mg until relapse (Nordic), 200 mg until 4 weeks after last cycle (HOVON) and 100 mg during 48 weeks (8 cycles) (Turkey). Melphalan dose was 4 mg/sqm day 1-7 every 4 weeks in 6 cycles (Italy), 0,25 mg/kg day 1-4 every 6 weeks below 65 years and 0,20 mg/kg above 65 years in 12 cycles (France), 0,25 mg/kg day 1-4 in cycles every 6 weeks until plateau phase (Nordic), 0, 25 mg/kg day 1-5 every 4 weeks in at least 8 cycles (HOVON), 9 mg/sqm day 1-4 every 6 weeks in 8 cycles (Turkey). The French study in patients above 65 years and the Nordic study were blinded with placebo.

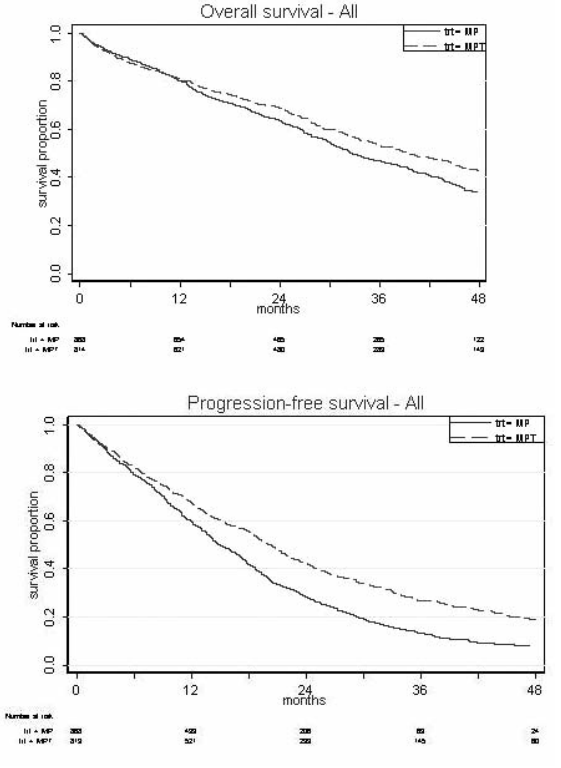

Figure.

Results. We analyzed 1682 patients, 868 in the MP arm and 814 in the MPT arm. Median OS was 32.7 (95% CI 30.4-36.5) months in the MP arm and 39.3 (35.6-39.0) months in the MPT arm. Median PFS was 14.9 (14.0-16.6) months in the MP arm and 20.4 (18.8-21.6) months in the MPT arm. Overall hazard ratio of MPT compared to MP was 0.82 (0.66-1.02) for OS and 0.67 (0.55-0.80) for PFS when a random effects model was used. Test of heterogeneity between the studies was statistically significant for OS (P=0.26) and for PFS (P=0.23). *Conclusions.* The meta analysis demonstrates a significant effect on progression free survival and a close to significant difference in OS when thalidomide is added to melphalan and prednisone in first line treatment to elderly patients with multiple myeloma.

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A PROSPECTIVE RANDOMIZED TRIAL OF BORTEZOMIB-MELPHALAN-PREDNISONE-THALIDOMIDE FOLLOWED BY CONTINUOUS BORTEZOMIB-THALIDOMIDE FOR INITIAL THERAPY OF MULTIPLE MYELOMA: EFFECT OF AGE AND CO-MORBIDITIES

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Background In elderly patients with newly diagnosed multiple myeloma (MM) bortezomib-melphalan-prednisone (VMP) and melphalan-prednisone-thalidomide (MPT) are now regarded as the new standards of care. *Aims.* To compare VMP-thalidomide followed by continuous bortezomib-thalidomide (VMPT-VT) with VMP alone as induction therapy. Primary end point was progression-free-survival (PFS). *Methods.* Between May, 2005 and January, 2009, 511 patients aged ≥ 65 years were randomized to receive VMPT-VT (N=254) or VMP (N=257). In the VMPT-VT arm patients received bortezomib 1.3 mg/m^2 days 1,4,8,11,22,25,29,32 in cycles 1-4 and days 1,8,22,29 in cycles 5-9; melphalan 9 mg/m^2 plus prednisone 60 mg/m^2 days 1-4 and thalidomide 50 mg days 1-42 for nine 6-week cycles, followed by VT therapy (bortezomib 1.3 mg/m^2 days 1, 15, thalidomide 50 mg/day). In the VMP arm patients received the same schedule without any further therapy. To evaluate if the treatment regimen could be further optimized by decreasing the toxicity while maintaining efficacy, in March 2007 the protocol was amended and both bortezomib schedules were reduced to once-weekly infusion.

Table.

	VMPT-VT (n = 250)	VMP (n = 253)	*P* Value (VMPT-VT vs VMP)
CR	38%	24%	<.001
≥ VGPR	59%	50%	.03
3-year PFS	56%	41%	.008
3-year OS	89%	87%	.77

Results. 503 patients were evaluable: 250 were assigned to receive VMPT-VT and 253 to VMP. Both arms were well balanced for baseline characteristics. Response rates were superior in the VMPT-VT group as compared to VMP: ≥VGPR rate of 59% vs 50% (P=.03) and CR of 38% vs 24% (P<.001), respectively. VT therapy was able to increase response rate in 11% of evaluable patients. After a median follow-up of 23.4 months, the 3-year PFS was 56% and 41% (P=.008), and the 3-year overall survival (OS) was 89% and 87% (P=.77) in the VMPT-VT group and in the VMP group, respectively. VMPT-VT induced a higher incidence of grade 3-4 neutropenia (38% vs 28%, P=.02) and cardiac complications (10% vs 5%, P=.04); the incidence of grade 3-4 peripheral neuropathy (PN) was 8% in VMPT and 5% in VMP (P=.19). During maintenance therapy with VT, the most relevant toxicity was PN (4%), other serious adverse events were less than 1.5%. 133 patients were older than 75

years, 65 in the VMPT-VT group and 68 in the VMP group. In patients ≥75 years of age, response rates and PFS were similar in VMPT-VT and VMP patients: ≥VGPR was 49% vs 41% (P=.35) and CR 31% vs 29% (P=.72), respectively; the 2-year PFS was 56% in the VMPT-VT and 53% in the VMP group (P=.49). In patients ≥75 years, the incidence of non-hematologic events (59% vs 31%, P=.001) cardiologic events (18% vs 4%, P=.01), and treatment discontinuation for toxicity (31% vs 16%, P=.04) were higher for VMPT-VT as compared to VMP. *Conclusion.* These results indicate that: 1. VMPT-VT was significantly superior to VMP; 2. cardiologic toxicity may reduce the efficacy of the VMPT-VT regimen in elderly frail patients.

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IMMUNOPHENOTYPIC RESPONSES CAN BE ACHIEVED IN ELDERLY MULTIPLE MYELOMA PATIENS TREATED WITH NOVEL AGENTS

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Introduction: Recent data suggest that the immunophenotypic response is one of the most relevant prognostic factors in MM patients treated with conventional therapy plus ASCT. However, so far the value of MRD investigations in the context of novel agents had not been explored, especially in the elderly population. *Aims.* To assess the frequency and the prognostic value of the multiparameter flow cytometry (MFC) remission status after induction therapy in elderly newly diagnosed non-transplanted MM patients. *Methods.* A total of 153 patients were evaluable for this analysis. All of them uniformly treated according to the Spanish GEM05>65y protocol (two arms of six cycles of Bortezomib/Melphalan/Prednisone (n=79) or Bortezomib/Thalidomide/Prednisone (n=74) supplemented with maintenance therapy). The median follow-up was 24 months and the EBMT criteria were used for response evaluation. Patients were defined to be in immunophenotypic response when myelomatous plasma cells (MM-PCs) were undetectable by MFC or when less than one phenotypically aberrant PC was detected among 105 cells analyzed. *Results.* After 6 induction cycles, residual MM-PC were undetectable or present at a frequency bellow 10^{-5} in 34 out of the 153 cases analyzed (22%), and thus qualified as MFC responses (MFCr); this frequency being similar in the VMP and VTP induction arms (24% vs. 19%, P=.4). Median time to progression (TTP) in these patients has not been reached while in the remaining 131 cases (78%) was 31 months (P<.001). A trend for longer overall survival (OS) was also seen in patients with MFCr compared to the rest of the cases (P=.07). MFC also afforded prognostic information within the 48 patients who achieved complete remission (CR) by immunofixation (IFx). The 2-year TTP rates were 100% in patients achieving MFCr (n=25) versus 70% in those who didn´t achieved (n=23;P=.03), and the respective 2-year OS rates were 100% versus 80% (P=.06). Moreover, we found that the better the quality of the response the longer the TTP, with 2-year rates of 100%, 83%, 57% and 36% for MFCr, CR, nCR+PR and less than PR, respectively;P<.001. Discordant results between MFC and IFx response status were found in 23 patients (21%), 7 being IFx+/MFC- and 16 IFx-/MFC+. Strikingly, in all 7 cases IFx+/MFC- the M-component became negative by IFx in subsequent analysis, consistent with the long half-life of some immunoglobulins in patients achieving an optimal response by MFC. In contrast, IFx-/MFC+ cases showed a tendency to early reappearance of the M-component (6/16;38%). Finally, the multivariate model selected the immunophenotypic response as the most potent marker for predicting PFS (hazard ratio -HR=33.6; P=.04) followed by highly abnormal baseline sFLC ratio (<0.02 or >10;HR=4.1;P=.007) and high% of MM-PC in S-phase (>2%;HR=3.4;P=.01), whereas CR failed to reach significance (HR=2.8;P=.15). *Conclusions.* Our results show that patients with immunophenotypic response display a significantly longer PFS, clearly superior to that of patients in CR by IFx, and that the achievement of an immunophenotypic response should be a goal even in elderly MM patients (non-transplant candidates) in the era of novel agents, confirming that the better the quality of response the longer the survival.

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ACHIEVEMENT OF COMPLETE REMISSION IS A STRONG PROGNOSTIC FACTOR IN 895 ELDERLY MYELOMA PATIENTS TREATED WITH MELPHALAN-PREDNISONE BASED-REGIMENS: RESULTS OF 3 MULTICENTER ITALIAN TRIALS

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Background. several studies, mostly in the context of autologous stem cell transplantation, demonstrate that a better tumor reduction significantly correlates with longer survival or at least progression-free survival (PFS) in multiple myeloma (MM) patients. In elderly patients, treated with conventional chemotherapy, complete remission (CR) was a relatively rare event since new drugs has been added to standard melphalan and prednisone (MP). *Aims.* to validate the prognostic value of CR in a large group of elderly newly diagnosed MM patients treated with MP based-regimens. *Methods.* 895 patients with newly diagnosed MM, aged ≥ 65 years (or younger but considered not eligible for high-dose chemotherapy), enrolled in the GISMM-2001, RV-MM-PI-026 and MM0305 multicenter Italian trials were analyzed. Patients received MP (n=164), MP plus thalidomide (MPT, n=167), MP plus lenalidomide (MPR, n=53), MP plus bortezomib (VMP, n=257) or MP plus bortezomib and thalidomide (VMPT-VT, n=254). Median number of cycles administered was 6 (range 1-9). Best response assessment was available in 847 patients. *Results.* the best response was CR in 195 patients, very good partial response (VGPR) in 171 patients and partial response (PR) in 297 patients. After a median follow-up of 51 months, 4-year progression-free-survival (PFS) was 62% in patients who obtained CR, 28% in those who achieved VGPR and 28% in patients who achieved partial response (PR) only (P<0.0001). Four-year overall survival (OS) was significantly higher in patients obtaining CR compared to VGPR or PR (79% versus 52% versus 54%, P<0.0001). At a landmark analysis performed at 6 months after enrolment (n=644) PFS and OS were significantly better in patients who achieved CR compared to patients with VGPR or PR (P<0.0001). Subgroup analysis was performed according to age, International Staging System (ISS) stage and treatment regimen. The benefit of achieving CR on PFS was confirmed in patients younger than 75 years (P<0.0001) and older (P<0.0001); in patients with ISS stage I (P=0.018), stage II (P<0.0001) and stage III (P<0.0001) and in patients treated with bortezomib-based regimen (VMP, P=0.003) or immunomodulatory agents (MPT and MPR, P=0.002) or bortezomib plus immunomodulatory agents (VMPT-VT, P=0.0004). Similarly, CR achievement was correlated to a significant increase in OS in patients younger (P=0.001) and older than 75 years (P=0.015) and in patients with ISS stage II (P=0.003) and III (P=0.006). By subgroup analysis of treatment regimen, the impact of CR on OS was confirmed both for patients treated with MPT and MPR (P=0.019) and with VMPT-VT (P=0.019). *Conclusions.* in elderly MM patients treated with MP-based regimens, CR achievement is a strong prognostic factor regardless of age, ISS stage and treatment regimen.

Table.

	CR N=195	VGPR N=171	PR n=297	CR vs VGPR vs PR p value
4-year PFS	62%	28%	28%	p<0.0001
4-year OS	79%	52%	54%	p<0.0001

Aggressive non-Hodgkin's lymphoma

0571

A GENETIC VARIANT OF MLH1, A GENE INVOLVED IN DNA MISMATCH REPAIR, IS AN INDEPENDENT PREDICTOR OF OVERALL SURVIVAL IN DIFFUSE LARGE B-CELL LYMPHOMA TREATED WITH R-CHOP

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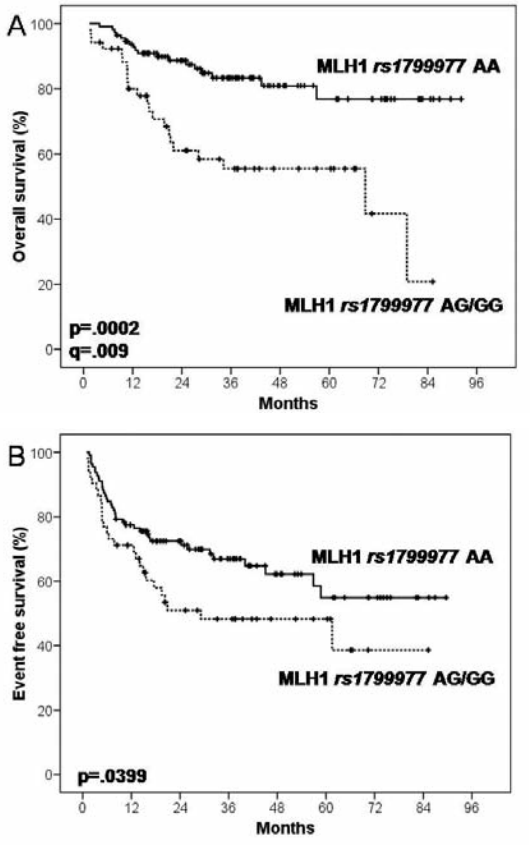
Background. Several drugs utilized in diffuse large B cell lymphoma (DLBCL) treatment rely on DNA damage for tumor killing. Host genetic variability in genes repairing DNA damage may affect response to drugs and prognosis. *Aims.* We verified the impact of DNA repair genes SNPs on prognosis of R-CHOP treated DLBCL. *Methods.* The study was based on 163 consecutive DLBCL treated with R-CHOP and provided with a prospectively collected dataset. At diagnosis, age >60 years was observed in 104/163 (63.8%) cases, ECOG PS >1 in 21/163 (12.9%), extranodal sites >1 in 41/163 (25.2%), Ann Arbor stage III-IV in 85/163 (52.1%), bulky in 46/163 (28.2%), LDH elevation in 75/163 (46.0%), IPI >2 in 55/163 (33.7%). Median follow-up was 48 months. Thirty-five SNPs from 18 genes were analyzed on patients' germline DNA. These included SNPs affecting: i) mismatch repair (MLH1rs1799977/rs1800734); ii) base excision repair (XRCC1rs1799782/rs25487, OGG1rs1052133); iii) nucleotide excision repair (ERCC1rs3212986, ERCC2rs1052555/ rs13181/rs1799793/ rs238406, ERCC4rs1800067/rs3136038, ERCC5rs17655, ERCC6rs2228528/rs2228529/rs3793784, XPArs1800975, XPCrs222799/ rs2228000/rs2607775/rs2228001); iv) double strand break repair (BRCA1rs49868507rs17999507rs799917, BRCA2rs144848, LIG4rs1805388, XRCC2rs3218536, XRCC3rs17997947rs861539, XRCC4rs1805377, XRCC6rs5751129/rs132788); and v) direct reversal (MGMTrs16906252/rs2308321/rs12917). Clinical endpoints were overall survival (OS) and event free survival (EFS). Informed consent was obtained.



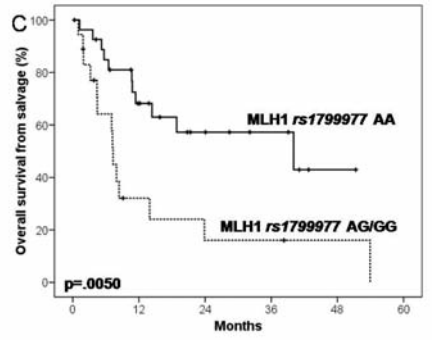


Figure.

Results. Univariate analysis controlled for multiple comparisons identified MLH1rs1799977 as the sole DNA repair SNP predicting OS in R-CHOP treated DLBCL. Patients carrying the MLH1rs1799977 AG/GG genotype displayed an increased risk of death (HR:3.23; 4-years OS: 55.5%) compared to AA carriers (4-years OS: 80.9%) (P=.0002; q=.009; Fig.1A). Multivariate analysis selected MLH1rs1799977 (HR:3.14; P=.0004) as an independent predictor of OS, along with IPI (HR:1.38; P=.0377) and bulky disease (HR:2.56; P=.0044). Accordingly, when combined to IPI, MLH1rs1799977 refined the clinical prognostication of DLBCL. The poor prognosis heralded by MLH1rs1799977 AG/GG genotype in DLBCL is due to first and second line treatment failure. Patients carrying the MLH1rs1799977 AG/GG genotype displayed an increased risk of failing R-CHOP (HR:1.66; 4-years EFS: 48.3%) compared to AA carriers (4-years EFS: 62.2%) (P=.0399; Fig.1B). Multivariate analysis identified MLH1rs1799977 (HR:1.66; P=.0498) as an independent predictor of EFS, along with IPI (HR:1.61; P<.0001) and bulky disease (HR:1.84; P=.0215). Also, patients carrying the MLH1rs1799977 AG/GG genotype displayed an increased risk of failing second line platinum-based regimens (HR: 3.04; 4-year OS from salvage: 16.0%) compared to AA carriers (4-year OS from salvage: 57.3%) (P=.0050; Figure 1C). By bivariate analysis, MLH1rs1799977 predicted OS from salvage independent of having (P=.0020) or having not (P=.0480) consolidated with SCT. *Conclusions.* MLH1rs1799977 is an independent predictor of survival in DLBCL treated with R-CHOP. The biologic plausibility of this association is supported by four lines evidence: i) MLH1rs1799977 is a nonsynonymous SNP causing the I219V amino acidic substitution on MLH1, a gene of the mismatch repair pathway; ii) in silico, MLH1rs1799977 is predicted to have deleterious consequences; iii) *in vitro*, the G variant allele of MLH1rs1799977 associates with reduced MLH1 protein expression; iv) loss of MLH1 in tumor cells is known to induce refractoriness to doxorubicin and platinum compounds. Consistently, DLBCL carriers of the MLH1rs1799977 AG/GG genotypes displayed poor OS possibly due to altered MLH1 expression.

0572

ROMIDEPSIN EXPERIENCE IN 317 PATIENTS WITH T-CELL LYMPHOMAS

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Background. Romidepsin is a novel pan-histone deacetylase (HDAC) inhibitor recently approved for the treatment of cutaneous T-cell lymphoma (CTCL) in patients with one prior systemic therapy. *Aims.* Evaluate efficacy and safety of romidepsin in patients with CTCL and peripheral T-cell lymphoma (PTCL). *Methods.* 317 patients received single-agent romidepsin in 3 phase 2, multicenter, international studies: GPI-04-0001 enrolled patients with CTCL; GPI-06-0002 patients with PTCL; and NCI 1312 patients with CTCL or PTCL. The primary endpoint for the CTCL studies was overall response rate (ORR) assessed by a composite endpoint including skin, blood, lymph nodes, and viscera. The primary endpoint for PTCL studies was complete response (CR) rate. Duration of response (DOR) and safety were evaluated for all patients. *Results.* Both populations were heavily pretreated (median 2-4 prior therapies) and represented patients with advanced disease. Romidepsin was active in CTCL and PTCL with ORRs of 34% and 38%, CR rates of 6% and 15%, and median DOR of 13.7-15 months and 10 months, respectively. For CTCL, the most common treatment-related adverse events (AEs) for all cycles were nausea (67%; 3% ≥grade 3), fatigue (49%; 10% ≥grade 3), and vomiting (34%; 2% ≥grade 3). For PTCL, the most common were nausea (53%; 3% ≥grade 3), thrombocytopenia (44%; 21% ≥grade 3), and fatigue (43%; 7% ≥grade 3). Thrombocytopenia (all grades and ≥grade 3 events) and neutropenia (≥grade 3 events) occurred at a higher frequency in patients with PTCL compared with CTCL. These differences are likely related to reduced marrow reserves due to prior therapies and increased incidence of bone marrow involvement in PTCL. Discontinuation due to AE occurred in 28/167 (17%) and 28/150 (19%) CTCL and PTCL patients, respectively. *Conclusions.* These results represent one of the largest single-drug T-cell lymphoma data sets. Romidepsin treatment resulted in clinically meaningful and durable responses in CTCL and PTCL. Overall, the safety profile was similar across T-cell lymphomas. Romidepsin appears to be a promising new therapy for T-cell lymphoma patients.

Table.

	CTCL		PTCL	
	GPI-04-0001 (n=96)	NCI 1312 (n=71)	GPI-06-0002 (n=103)[a]	NCI 1312 (n=47)
Patient characteristics				
Mean age (range), years	57 (21-89)	56 (28-84)	59 (24-83)	60 (28-84)
Number of prior therapies, median (range)	4 (0-8)	3 (0-7)	2 (1-8)	4 (1-14)
ECOG performance status, n (%)				
0	50 (52%)	20 (28%)	35 (35%)	20 (43%)
1	46 (48%)	41 (58%)	51 (50%)	23 (49%)
2	0	10 (14%)	15 (15%)	4 (9%)
Disease stage/most common diagnosis at study entry, n (%)				
Stage ≥IIB (CTCL)	68 (71%)	62 (87%)	NA	NA
PTCL not otherwise specified	NA	NA	31 (30%)	29 (62%)
Angioimmunoblastic T-cell lymphoma	NA	NA	12 (12%)	7 (15%)
Anaplastic large-cell lymphoma[b]	NA	NA	10 (10%)	2 (4%)
Under review	NA	NA	42 (42%)	NA
Response				
ORR (CR + PR), n (%)	33 (34%)	24 (34%)	NA	18 (38%)
CR	6 (6%)	4 (6%)	NA	7 (15%)
Median (range) DOR, months	15 (1-20)	13.7 (1-76)	NA	10 (2-70)

ECOG, Eastern Cooperative Oncology Group; NA, not available; PR, partial response.
[a] Patients accrued to August, 2009. [b] ALK-1–negative.

0573

ANTI-CD22 IMMUNOCONJUGATE INOTUZUMAB OZOGAMICIN (CMC-544) + RITUXIMAB: CLINICAL ACTIVITY IN PATIENTS WITH RELAPSED/REFRACTORY FOLLICULAR OR 'AGGRESSIVE' LYMPHOMA

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Background. Inotuzumab ozogamicin (CMC-544) is a humanized anti-CD22 antibody conjugated to calicheamicin, a potent cytotoxic agent. CD22 is expressed on the majority of B cell non-Hodgkin lymphomas (NHL). CMC-544 has demonstrated single agent efficacy and good tolerability in heavily pre-treated and refractory patients with NHL. *Aims.* To assess clinical activity of CMC-544 combined with rituximab in patients with NHL, including: relapsed follicular lymphoma (FL), relapsed diffuse large B cell lymphoma (DLBCL), and refractory 'aggressive' NHL. *Methods.* Part 1 dose-escalation phase determined the maximum tolerated dose (MTD) of CMC-544 combined with rituximab to be 1.8 mg/m^2 q4w. In part 2, eligible patients had CD20$^+$/CD22$^+$ B cell NHL and prior rituximab. Patients with relapsed FL and DLBCL had ≤2 prior therapies, but were not refractory to rituximab-containing therapy. Patients with rituximab-refractory 'aggressive' NHL could have DLBCL, mantle cell lymphoma, or transformed FL, with no limit on prior therapies. Patients received 375 mg/m^2 rituximab IV on Day 1, followed by 1.8 mg/m^2 CMC-544 on Day 2 of each 28-day cycle for up to 8 cycles in the absence of disease progression. Safety, efficacy (ie, objective response rate [ORR], overall survival [OS], progression-free survival [PFS]), and pharmacokinetics were evaluated. *Results.* The study enrolled 119 patients, including 110 treated with CMC-544 at the MTD of 1.8 mg/m^2. Median age was 66 years (range: 20-85); 60% were male; 35% had 1 prior chemotherapy/immunotherapy regimen, 46% had 2, and 18% had ≥3; 73% had stage III/IV disease; 40% had elevated lactate dehydrogenase; and 19% had bulky disease (>7.5 cm). Common drug-related treatment-emergent adverse events (TEAEs) included thrombocytopenia (46%), nausea (44%), fatigue (40%), and increased aspartate aminotransferase (33%). Drug-related serious TEAEs were reported for 12 (10%) patients. Median follow-up was 16.7, 10.9, and 3.1 months for patients with FL, recurrent DLBCL, and 'aggressive' NHL treated at the MTD, respectively. Patients with relapsed FL (n=38) had an ORR of 84%; patients with relapsed DLBCL (n=40) had an ORR of 80%. One-year OS and PFS rates were 97% and 80% (median PFS not reached), respectively, for patients with relapsed FL. One-year OS and PFS rates were 79% and 56% (median PFS: 15.1 months), respectively, for patients with recurrent DLBCL. Response to prior therapy appears to be a major prognostic factor. Patients with rituximab-refractory 'aggressive' NHL (n=28) had a lower ORR (18%), with a median PFS of only 1.7 months. Maximal serum CMC-544 concentration (measured by total calicheamicin) was comparable across cycles (61-71 ng/mL). However, exposure (AUC) increased with each subsequent cycle (Cycle 1: 4,700 ng/mL; Cycle 2: 8,600 ng/mL; Cycle 3: 9,558 ng/mL); half-life also increased after Cycle 1. *Summary/Conclusions.* The combination of inotuzumab ozogamicin (CMC-544) plus rituximab has a safety and pharmacokinetic profile similar to that previously reported for CMC-544 alone. The ORR and PFS results indicate promising efficacy in patients with relapsed FL and DLBCL. Patients with rituximab-refractory 'aggressive' NHL had a low ORR and median PFS, consistent with their expected poorer prognosis. These encouraging results in patients with relapsed NHL support continued clinical development of this regimen.

0574

LENALIDOMIDE MONOTHERAPY IS CLINICALLY ACTIVE IN PATIENTS WITH RELAPSED/REFRACTORY DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL): A POOLED ANALYSIS OF DATA FROM 2 PHASE II STUDIES (NHL-002/003)

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Background. DLBCL is the most frequently diagnosed type of aggressive non-Hodgkin's lymphoma (aNHL). Despite recent advances failure-free survival remains at ~50% (Habermann JCO 2006), and prognosis remains dismal for those not cured with either R-CHOP or autologous stem cell transplantation (SCT). Lenalidomide is an immunomodulatory agent that demonstrates considerable clinical activity in the treatment of B-cell hematologic malignancies. Two phase II trials, NHL-002 (49 patients; Wiernik JCO 2008) and a larger scale, international study NHL-003 (217 patients) have been conducted in patients with relapsed/refractory aNHL of various histologies treated with single-agent lenalidomide at the schedule of 25 mg days 1-21 in a 28-day cycle. Here, we present the subset analysis of patients with DLBCL from the NHL-003 study, as well as pooled data from both studies evaluating the clinical utility of lenalidomide in patients with relapsed/refractory DLBCL (off-label). *Aims.* To assess the clinical activity and tolerability profile of lenalidomide monotherapy in patients with relapsed/refractory DLBCL. *Methods.* In both studies, patients with relapsed/refractory aNHL (defined as DLBCL, mantle cell lymphoma, transformed lymphoma, and follicular lymphoma grade 3), with measurable disease (≥2 cm), and ≥1 prior treatment, provided informed consent and received oral lenalidomide 25 mg once daily on days 1-21 of every 28-day cycle. In NHL-002, treatment continued for ≤52 weeks, whereas in NHL-003 treatment continued until disease progression. Overall response rate was the primary endpoint; secondary endpoints included response duration, progression-free survival (PFS), and safety. Histology and response were as per investigator assessment. *Results.* 26 patients from NHL-002 and 108 patients from NHL-003 had a DLBCL histology and were included in this analysis. For the pooled population of 134 patients, the median age was 66 years (21-87), median time from diagnosis was 2.3 years (0.3-21.1), and 82 patients (61%) were male. Patients failed a median of 3 prior regimens (1-10), and 52 (39%) patients had prior SCT. Lenalidomide monotherapy induced responses in 35 patients (26%), which included 12 patients (9%) who achieved a complete response (CR)/unconfirmed CR (CRu). Responses were observed in 10 of 62 patients (16.1%) refractory to their last prior therapy and in 15 of 52 patients (28.8%) with SCT. At a median 4.6-month follow-up median response duration was 6.0 months. Patients with a CR/CRu after lenalidomide achieved a median response duration lasting 10.4 months. Consistent with other studies of lenalidomide most common grade 3 and 4 adverse events were reversible neutropenia (22.4% and 13.4%), thrombocytopenia (15.7% and 5.2%), and anemia (6% and 0.7%). *Conclusions.* This analysis of 134 patients pooled from 2 phase II studies shows that single-agent lenalidomide has clinical activity and can achieve durable responses in patients with relapsed/refractory DLBCL. Patients with a CR/CRu achieved particularly promising response durations. The data also suggest that among patients with DLBCL there may be sub-populations for whom lenalidomide may provide a particular benefit. Additional studies are underway to explore patient subsets that are more likely to respond to lenalidomide, and to evaluate lenalidomide in combination with other agents currently used to treat aNHL.

0575

SUSTAINED SURVIVAL ADVANTAGE AFTER A MEDIAN FOLLOW-UP OF 5 YEARS FOR IMMUNO-CHEMOTHERAPY (R-MCP) VERSUS CHEMOTHERAPY ALONE (MCP) IN ADVANCED FOLLICULAR LYMPHOMA - UPDATE OF THE OSHO#39 TRIAL

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Background. When we presented our results of R-MCP immunochemotherapy for follicular lymphoma at the 2006 ASH meeting, this was the first randomized phase III study ever demonstrating an overall survival advantage of immunochemotherapy over chemotherapy alone. Now we are now able to report much more mature data with a median follow up of 60 months. *Methods.* After informed consent previously untreated patients with advanced stage, symptomatic CD 20-positive indolent NHL and mantle cell lymphoma (n=358) were randomized to receive either MCP-chemotherapy (mitoxantrone 8 mg/m² d1+2, chlorambucil 3x3 mg/m² d 1-5, prednisolone 25 mg/m² d 1-5 x 8 q 4 weeks) or MCP + rituximab (375 mg/m² d -1) followed by interferon maintenance treatment (3 x 4.5 mioIU per week) for responding (CR, PR) patients. Here we report the 60 months follow up results of the follicular lymphoma patients (grade 1+2), who represented the majority of patients and for whom the sample size was calculated, so this is not a subgroup analysis. Study endpoints included overall and complete response rate (RR + CR), progression free survival (PFS), event free survival (EFS), time to next treatment (TTNT), overall survival (OS) and toxicities. *Results.* Concerning toxicities there was no striking difference. For the FL - ITT population the treatment results are as follows: R-MCP (n=105) MCP (n=96) p - value. Response rate 92,4% 75% .0009. Complete responses 49,5% 25% .0004. PFS median 86 mo. 35 mo. < .0001 .PFS 5 y 65% 33%. EFS median 86 mo. 27 mo. .0001. EFS 5 y 63% 30%. TTNT median n.r. 29 mo. <.0001. No retreatment at 5 y 55% 31%. OS median n.r. 108 mo. .0278. OS 5 y 86% 74%. (PFS=progression free survival, EFS=event free survival, TTNT= time to next treatment, OS=overall survival). *Conclusions.* Concerning all end points rituximab plus MCP remains to be significantly superior to MCP alone: after a median follow-up of now 5 years we can demonstrate that an improvement in induction treatment (CR-rate doubled) results in a highly significant advantage concerning survival parameters: PFS and EFS as well are doubled and OS is clinically and statistically superior too.

Acute myeloid leukemia - Models

0576

CELL OF ORIGIN INFLUENCES LEUKEMIA STEM CELL PHENOTYPE

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MLL-fusion proteins can transform either hematopoietic stem cells (HSC) or granulocyte macrophage progenitors (GMP) into leukemia stem cells (LSC). We hypothesized that different cells of origin would generate LSC with different properties. First, we assessed kinetics of AML development through introduction of MLL-AF9 into HSC or GMP isolated from mice that express luciferase under control of the ubiquitin promoter. The expansion of leukemias was monitored using *in vivo* bioluminescent imaging at multiple time points after transplantation. We observed almost linear kinetics of accumulation of leukemic cells when AML was initiated from 3×10^3 Lin-Kit+sca1+ cells, and there was a 3 to 4-week delay in accumulation of leukemic cells when AML was initiated from GMP. To assess leukemic transformation of individual cells we transduced HSC and GMP with MLL-AF9 or control retroviruses, then sorted single-cells expressing MLL-AF9 (GFP+). Both the infected HSC and GMP could be serially replated for over 9 passages. Upon transplantation into syngeneic mice, 86.3% (n=22) of HSC:MLL-AF9 single cell derived clones (SCC) induced AML with a median latency of 61 days, while 33.3% of GMP:MLL-AF9 SCC induced AMLs with median latency of 100 days. Given the apparent increased aggressiveness of HSC-derived leukemias we assessed drug response. Treatment with Etoposide reduced the spleen weights in mice transplanted with HSC-derived AML to a lesser extent (28%) than in mice transplanted with GMP-derived AML (88%) suggesting the cell of origin influences drug response. Immunophenotype analysis of the resultant leukemias demonstrated that long-term repopulating HSC and GMP-derived leukemias were quite similar, with a GMP-like (LGMP) population enriched for LSC in both cases. Gene expression analysis demonstrated that globally the LGMP isolated from HSC derived AML and from GMP derived AML were similar to each other, but possessed specific gene expression programs reminiscent of their cell of origin (HSC or GMP). For example Evi1, Jun, Jund, and Fos oncogenes were highly expressed in HSC and the AML that arose from HSC, but were expressed at low levels in GMP or the AML that arose from GMP. The gene expression program that distinguished LGMP-HSC from LGMP-GMP was found to correlate with a gene expression program in human MLL-rearranged AML associated with a poor clinical outcome in three independent MLL-rearranged AML data sets. Computational modeling of activated signaling pathways predicted the Wnt/Ctnnb1 pathway is more active in LSC arising from HSC than those that arose from GMP. Additionally, Ctnnb1 target genes were expressed at higher levels in LSC originating from HSC, and Ctnnb1 reporter activity was higher in MLL-AF9 transformed HSC as compared to MLL-AF9 transformed GMP. Finally, western blotting analysis demonstrated higher levels of Ctnnb1 in LSC originating from HSC than those originating from GMP. Altogether, these data indicate that the cell of origin of AML can influence the genetic program of the fully developed LSC, and thus might account for some of the heterogeneity in human leukemias and their clinical outcome. Furthermore, suppression of Ctnnb1 might be a rational approach in AML.

0577

DEVELOPMENT OF A MOUSE MODEL OF ONCOGENIC COOPERATION IN DS-AML AND IDENTIFICATION OF HUMAN CHROMOSOME 21 GENES THAT FACILITATE LEUKEMOGENESIS

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Background. Children with Down syndrome (DS) have a 500-fold increased risk of Acute Megakaryoblastic Leukemia (DS-AMKL). In addition to trisomy 21, DS-AMKL blasts typically harbor somatic mutations in the gene encoding GATA-1, an essential transcriptional regulator of erythroid and megakaryocytic differentiation. In every case, the GATA1 mutations lead to a block in expression of the full-length protein, but allow for translation of a shortened isoform named GATA-1s, which lacks the N-terminal transcriptional activation domain. Recent studies have shown that genetic abnormalities affecting JAK2, JAK3 and MPL, which lead to constitutive activation of JAK/STAT pathway, is another feature of AMKL. *Aims.* Whereas the impact of genetic mutations in GATA1, JAK2 and MPL is relatively well known, the specific effects of trisomy 21, the initiating event in DS leukemogenesis, is still under investigation. Here, we used two different models of DS-AMKL to identify specific genes on human chromosome 21 (Hsa21) that participate in leukemogenesis: human DS-AMKL cell lines and the Ts1Rhr mouse strain, which is trisomic for only 33 orthologs of Hsa21 genes. *Methods.* We comprehensively analyzed hematopoiesis in progressively more complex models of DS-AMKL: this included Ts1Rhr mice, Ts1Rhr/Gata-1s mutant mice, and recipients of Ts1Rhr/Gata-1s/ hematopoietic progenitors expressing MPL W515L. In parallel, we performed a shRNA screen in the CMY and CMK DS-AMKL cell lines to identify specific Hsa21 genes implicated in megakaryocyte proliferation, survival and/or differentiation. Genes identified in this screen were then evaluated for expression and activity in our *in vivo* model of DS-AMKL. *Results.* Ts1Rhr mice develop a progressive myeloproliferative disease characterized by a thrombocytosis, increased number of megakaryocytes, extramedullary hematopoiesis and alterations in the HSC/progenitor compartment. By breeding Ts1Rhr mice with Gata1s knock-in mice, we discovered that Gata-1s expression transiently cooperates with the trisomy to induce a prolonged thrombocytosis. However these double transgenic mice failed to develop AMKL. Next we overexpressed the MPL W515L mutant in Ts1Rhr/Gata-1s hematopoietic progenitors and transplanted these cells to lethally irradiated recipients. We observed rapid development of a fatal megakaryocytic disorder that was accompanied by profound marrow myelofibrosis and the presence of immature megakaryoblasts. Of note, expression of MPL W515L in either Ts1Rhr or Gata-1s alone failed to cause a similar disease. Thus, these 3 genetic events are necessary and sufficient for AMKL *in vivo*. Analysis of candidate genes that were selected from our human CMY and CMK cell line screening revealed that ERG, DYRK1A, CHAF1B and HLCS likely all play a role in etiology of DS-AMKL. *Summary*. Here we report that the partially trisomic Ts1Rhr mice display progressive alterations of the megakaryocytic compartment, that mutagenesis of Gata1 in the Ts1Rhr background exacerbates the phenotype, and that combining mutant Gata-1, trisomy for Hsa21 orthologs and an activating mutation of MPL leads to a fulminant megakaryocytic leukemia with myelofibrosis. Moreover, we identify four Hsa21 genes that are required for the phenotype of human megakaryocytic cell lines that also function in the murine AMKL model, emphasizing a likely role of those genes in predisposition to DS-AMKL.

0578

A CONDITIONAL KNOCK-IN ALLELE OF THE TYPE A CYTOPLASMIC NUCLEOPHOSMIN MUTATION COOPERATES WITH A NOVEL SLEEPING BEAUTY TRANSPOSON TO CAUSE ACUTE MYELOID LEUKAEMIA IN MICE

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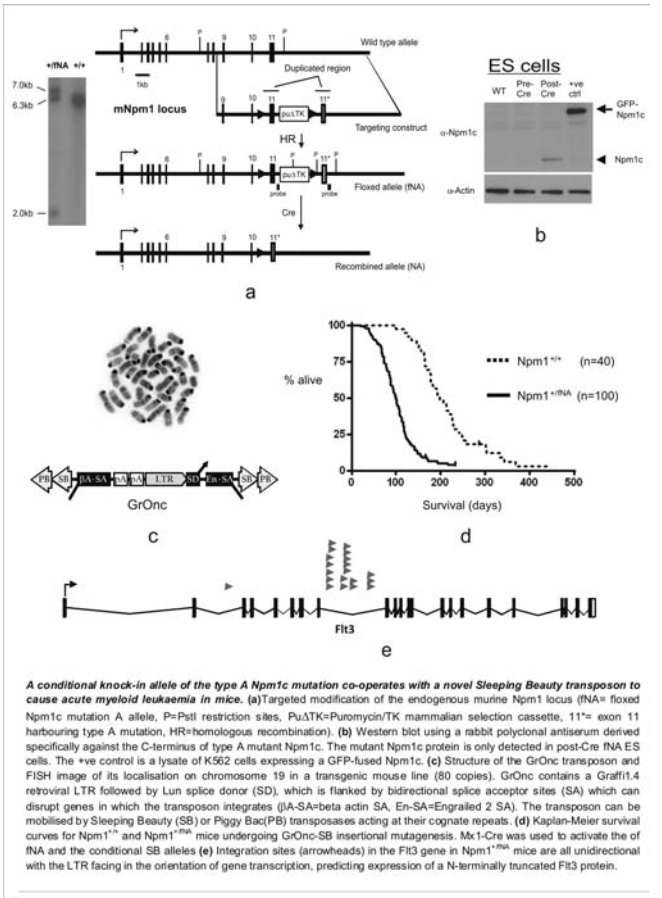
Mutations in the gene for nucleo-cytoplasmic shuttle protein Nucleophosmin (NPM1) are found in up to 60% of acute myeloid leukaemia (AML) with normal karyotype. Nearly all such mutations occur within the last exon of the NPM1 gene and are associated with aberrant localisation of NPM1 protein to the cytoplasm (NPM1c mutations). However, the mechanism through which NPM1 mutations cause AML remains unknown. To address this, we generated a conditional knock-in mouse model of type A mutation which represents 80% of all human NPM1 mutations. Our floxed allele (Npm1fNA) leaves the native Npm1 locus largely intact, yet enables the expression of mutant Npm1c after Cre-recombination (Figure 1a,b). Heterozygous germline activation of this allele by Cre was universally lethal before embryonic day 8 (Observed 0, Expected 34; P<0.00001). By contrast, heterozygous activation of the allele in the haematopoietic system through the action of Mx1-Cre was associated with an expansion in the bone marrow mature myeloid compartment and increased whole bone marrow serial re-plating ability in methylcellulose assays. Npm1+/fNA, Mx1-Cre double transgenic mice had a significantly increased incidence of acute myeloid and lymphoid leukaemias compared to single transgenic controls (20/43 vs 7/45, p =0.0024). However, Npm1+/fNA leukaemias developed after a median of 493 days (264-802) suggesting that additional mutations are required

for leukaemogenesis. In order to identify genes that can cooperate with Npm1c to cause AML we generated a transgenic mouse line carrying the novel transposon, GrOnc, that can be mobilised by Sleeping Beauty and PiggyBac transposases and can both activate and disrupt genes (Figure 1c). Using Mx1-Cre and a conditional Sleeping Beauty line we performed insertional mutagenesis screens in Npm1+/cNA and in Npm1+/+ mice. Npm1+/+ mice (n=40) developed lymphoid leukaemias/lymphomas after a median of 197 days. By contrast, in Npm1+/cNA mice (n=100), leukaemias were mainly myeloid and their onset was accelerated (median 99 days, P<0.0002). Initial analysis of transposon integration sites from 33 Npm1+/+ and 83 Npm1+/cNA mice, identified common integration sites (CISs) in known and novel cancer genes in both types of mice. Additionally, several CISs were unique to Npm1+/cNA mice including many in genes not previously described to cooperate with Npm1c. Amongst other genes we identified recurrent integrations in Flt3, mutations of which occur frequently in human Npm1c-positive AML, validating this approach and the significance of other loci hit in this screen. In summary, we have developed a novel knock-in mouse model of the type A Npm1c mutation which develops AML with features reminiscent of the human disease. Furthermore, using a novel transposon we have identified known and novel cancer genes that can cooperate with Npm1c to cause AML.



A conditional knock-in allele of the type A Npm1c mutation co-operates with a novel Sleeping Beauty transposon to cause acute myeloid leukaemia in mice. **(a)**Targeted modification of the endogenous murine Npm1 locus (fNA= floxed Npm1c mutation A allele, P=PstI restriction sites, PuΔTK=Puromycin/TK mammalian selection cassette, 11*= exon 11 harbouring type A mutation, HR=homologous recombination). **(b)** Western blot using a rabbit polyclonal antiserum derived specifically against the C-terminus of type A mutant Npm1c. The mutant Npm1c protein is only detected in post-Cre fNA ES cells. The +ve control is a lysate of K562 cells expressing a GFP-fused Npm1c. **(c)** Structure of the GrOnc transposon and FISH image of its localisation on chromosome 19 in a transgenic mouse line (80 copies). GrOnc contains a Graffi1.4 retroviral LTR followed by Lun splice donor (SD), which is flanked by bidirectional splice acceptor sites (SA) which can disrupt genes in which the transposon integrates (βA-SA=beta actin SA, En-SA=Engrailed 2 SA). The transposon can be mobilised by Sleeping Beauty (SB) or Piggy Bac(PB) transposases acting at their cognate repeats. **(d)** Kaplan-Meier survival curves for Npm1$^{+/+}$ and Npm1$^{+/fNA}$ mice undergoing GrOnc-SB insertional mutagenesis. Mx1-Cre was used to activate the of fNA and the conditional SB alleles **(e)** Integration sites (arrowheads) in the Flt3 gene in Npm1$^{+/fNA}$ mice are all unidirectional with the LTR facing in the orientation of gene transcription, predicting expression of a N-terminally truncated Flt3 protein.

Figure 1. Npm1c cooperates with Sleeping Beauty to cause AML.

0579

MEIS1 CONTROLS SUSCEPTIBILITY TO MN1-INDUCED LEUKEMIC TRANSFORMATION

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Background. Leukemia stem cells have been identified in human acute myeloid leukemia and several mouse leukemia models by prospective isolation of phenotypically characterized cells. It has become clear that the phenotype of leukemia stem cells does not necessarily reflect the cell of origin in which the transforming events occurred. For several oncogenes it has been shown that a spectrum of hematopoietic stem and progenitor cells rather than a specific lineage can be transformed. *Aims.* We evaluated which normal hematopoietic cell might be the cell of origin in MN1-induced leukemias, and which genetic factors might influence permissiveness to transformation. *Methods.* We employed retroviral infection of single sorted hematopoietic progenitor cells, colony-forming cell (CFC) and mouse bone marrow transplantation assays, gene expression profiling, and chromatin-immunoprecipitation (ChIP) sequencing. We found that (1) common myeloid progenitors (CMP), but not granulocyte-macrophage progenitors (GMP) can be transformed by MN1, (2) GMP transformation can be achieved by cotransduction of MN1 and MEIS1, (3) MN1 and MEIS1 co-regulate a large set of putative direct target genes, and (4) transcriptional repression of MEIS1 target sites by a M33-MEIS1 fusion protein prevents MN1-induced transformation. *Results.* Retroviral infection of single cell-sorted common-myeloid (CMP) and granulocyte-macrophage progenitors (GMP) with MN1 demonstrated that CMPs (n=16) but not GMPs (n=11) could be transformed by MN1 as evidenced by the capacity to serially replate in CFC assays and to induce leukemia in mice (P<.001). Moreover, bulk infections of up to 4000 GMPs with MN1 did not transform these cells. To identify pathways explaining the differences in transformation susceptibility between CMPs and GMPs, we compared gene expression profiles of MN1 leukemias, normal CMPs, and normal GMPs. FLT3, MEIS1, and HOXA10 were upregulated in MN1 leukemias and CMPs, but downregulated in GMPs. None of these factors could transform GMPs *in vitro* when co-infected with a control gene. However, GMPs were readily transformed by co-infection of GMPs with MN1 and MEIS1, but not FLT3, as evidenced by the ability to undergo more than 10 serial replatings in CFC assays. To further elucidate the mechanism of MEIS1-dependent transformation susceptibility of GMPs, we employed next-generation sequencing of chromatin immunoprecipitates and gene expression profiling of MN1 and MEIS1 to identify shared chromatin targets and genes coregulated by MN1 and MEIS1. Sixteen percent of MN1 targets overlapped with MEIS1 targets. We thus hypothesized that the transforming potential of MN1 relies on the transcriptional activity of MEIS1, and evaluated the leukemogenic potential of MN1 when coexpressed with a dominant negative form of MEIS1. Strikingly, coexpression of M33-MEIS1 in MN1 expressing cells prevented leukemic outgrowth of double transduced cells *in vivo*. *Conclusions.* Our data identify transcriptional states that determine transformation susceptibility of hematopoietic progenitor cells, and highlight the interactions of oncogene and susceptible cell. The requirement of MEIS1 as a cooperating gene in many different leukemia models reported previously highlights the importance of developing novel therapeutic strategies which target MEIS1 in leukemia.

0580

EXPRESSION OF THE CYTOPLASMIC NPM1 MUTANT (NPMC+) CAUSES THE EXPANSION OF HEMATOPOIETIC CELLS IN ZEBRAFISH

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Mutations in the human nucleophosmin (NPM1) gene are the most frequent genetic alteration in adult acute myeloid leukemia (AML) and result in aberrant translocation of this nucleolar phosphoprotein to cytoplasm (NPMc+). However, the underlying mechanisms leading to leukemogenesis remain unknown. To address this issue, we took advantage of the zebrafish model organism, which is characterized by rapid, external embryonic development and conserved primitive and definitive hematopoiesis. Perturbation of zebrafish hematopoiesis can be studied upon transient gene knock-down or overexpression through injection of antisense oligonucleotides (morpholinos) or mRNA, respectively. We found that zebrafish express two npm1 genes, both highly similar and orthologous to human NPM1, referred to as npm1a and npm1b. Both genes are ubiquitous and knock-down of each gene produces a reduction in myeloid cell numbers as shown by anti-mpx whole mount *in situ* hybridization (WISH). This effect is synergistic when both genes are knocked down, suggesting partial redundancy in their functions. Importantly, the reduction in myeloid cell number is specifically rescued by NPM1 expression, indicating functional conservation of NPM1 in zebrafish. When expressed in zebrafish, wild-type human NPM1 is nucleolar and NPMc$^+$ is cytoplasmic by confocal microscope analysis, as in human AML. Co-immunoprecipitation studies show that both forms of NPM1 interact with endogenous zebrafish Npm1a and Npm1b. Importantly, NPMc$^+$ dislocates both Npm1 proteins to the cytoplasm, as in human AML. Taken together, these data provide a strong rationale for testing the consequences of NPMc+ expression in the developing hematopoietic system of zebrafish embryos. WISH and FACS analysis in the Tg(pu.1:EGFP) transgenic line showed that the forced expression of NPMc$^+$ in zebrafish causes an increase in pu.1-positive primitive myeloid precursors at 19 hpf but not in mature mpx-positive and csf1r-positive myeloid cells at 24 hpf because of a p53-dependent apoptotic cell death response. In p53m/m embryos expressing NPMc+ no cleavage of caspase-3 is seen, and all three markers of primitive hematopoiesis show increased numbers of stained cells by WISH. We also investigated whether NPMc$^+$ expression results in perturbation of definitive hematopoiesis. To this end, we analyzed the number of hematopoietic stem cells (HSCs) in the ventral wall of the aorta, an hematopoietic site corresponding to the mouse Aorta Gonad Mesonephros, and the numbers of transient erythromieloid progenitors (EMPs) in the posterior blood island (PBI). Importantly, upon NPMc+ expression, increased numbers of c-myb-positive and cd41-positive cells were observed in the ventral wall of the aorta both in the presence and absence of a functional p53, suggesting that HSC number is increased. These results were confirmed by WISH, FACS and confocal analysis in the Tg(c-myb:EGFP) and in the Tg(cd41:EGFP) zebrafish lines. Furthermore, an expansion of EMPs was also noted, as shown by increased numbers of gata1$^+$/lmo2BRIGHT cell in the PBI by FACS and confocal microscope analysis. These results are highly relevant to human AML, where NPMc$^+$ is expressed in leukemia-initiating cells and in all myeloid lineages arising from the leukemic clone. The zebrafish model will thus provide an invaluable platform to dissect the developmental and cellular pathways affected by NPMc$^+$ expression.

Chronic myeloid leukemia - Biology

0581

ISOLATION AND KILLING OF CANDIDATE CML STEM CELLS BY ANTIBODY TARGETING OF IL1RAP

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Therapeutic strategies aiming for a permanent cure of chronic myeloid leukemia (CML) will require full eradication of Philadelphia chromosome positive (Ph+) CML stem cells. One such strategy is an antibody-based therapy directly targeting CML stem cells, in which the antibody mode of action is independent of the known resistance mechanisms causing CML stem cells to be unresponsive to tyrosine kinase inhibitor treatment. The major limitations for such developments have so far been the lack of a cell surface receptor distinguishing Ph$^+$ from Ph- CML stem cells. Here we used gene expression profiling to identify Interleukin 1 Receptor Accessory Protein (IL1RAP) as upregulated in CML CD34$^+$ cells and also in cord blood (CB) CD34$^+$ cells as a consequence of retroviral BCR/ABL1 expression. In agreement with the microarray gene expression data, we confirmed by FACS analysis that the IL1RAP protein indeed becomes upregulated on the cell surface of CB CD34$^+$ cells following retroviral P210 BCR/ABL1 expression. In the CML CD34$^+$CD38$^-$ population, containing both Ph$^+$ and Ph$^-$ CML stem cells, two populations of cells were identified: one exhibiting low/absent IL1RAP expression, and the other having higher IL1RAP expression. The IL1RAP-positive cell fraction constituted between 75% and 95% of the CML CD34$^+$CD38$^-$ cells (n=5), representing about one cell in 1,300 mononuclear cells. The more rare CD34$^+$CD38$^-$IL1RAP$^-$ cells represented only about one cell in 11,000 mononuclear cells. To test whether IL1RAP expression distinguishes normal (Ph$^-$) and leukemic (Ph$^+$) cells within the CML CD34$^+$CD38$^-$ cell compartment, we established a new protocol for conducting FISH on small numbers of sorted cells. By using this method, referred to as Flow-drop-FISH, we sorted cells directly into drops on slides to investigate their Ph-chromosome status. Interestingly, we found that the CML CD34$^+$CD38$^-$IL1RAP$^+$ cells were Ph$^+$ (99.9±0.2% Ph$^+$, n=5), whereas CML CD34$^+$CD38-IL1RAP- cells were almost exclusively Ph- (97.1±3.4% Ph-, n=5). By performing long-term culture-initiating cell assays on the two cell populations, we found that Ph+ and Ph- candidate CML stem cells could be prospectively separated. To test whether antibody-dependent cell-mediated cytotoxicity (ADCC) could be achieved using IL1RAP as a target, we generated a polyclonal anti-IL1RAP antibody. By using this antibody, we were able to provide proof of concept that IL1RAP can be used as a target on CD34$^+$CD38$^-$ candidate CML stem cells to induce cell death through ADCC in an NK-cell dependent manner. In summary, this study thus identifies IL1RAP as the first cell surface biomarker distinguishing Ph$^+$ from Ph$^-$ candidate CML stem cells and opens up a new avenue for exploring diagnostic and therapeutic strategies in CML.

0582

WHOLE-TRANSCRIPTOME SEQUENCING IN CHRONIC MYELOID LEUKEMIA AT DIAGNOSIS AND AT THE TIME OF PROGRESSION TO BLAST CRISIS REVEALS NOVEL GENE MUTATIONS

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Background. Philadelphia-positive (Ph$^+$) chronic myeloid leukemia (CML) is generally regarded as a genetically heterogeneous disease and therapy with Bcr-Abl inhibitors results in high response rates. Nevertheless, resistance may develop, especially in high Sokal risk patients, and progression to BC still represents a major concern. *Aims.* To perform a qualitative and quantitative survey of the whole transcriptome of Ph$^+$ CML cells at diagnosis and at progression in order to shed further light on the biological bases underlying high risk, as well as on the determinants of BC. *Methods.* We used massively parallel sequencing on a Solexa

Illumina Genome Analyzer to scan the transcriptome of a CML patient at the time of diagnosis, at the time of remission (major molecular response) and at the time of progression from chronic phase (CP) to lymphoid blast crisis (BC). Both custom scripts and published algorithms were used for read alignment against the human reference genome, for single nucleotide variant (SNV) calling, for identification of alternative splicings and fusion transcripts, and for digital gene expression profiling. Sanger sequencing was used for validation and for further screening of a preliminary additional subset of 10 CP CML patients at diagnosis (4 low, 3 intermediate and 3 high Sokal risk) and 10 BC CML patients. *Results.* Comparison of the SNVs identified in the diagnosis and relapse samples with the SNVs detected in the remission sample - representing inherited sequence variants not specific for the Ph+ clone - allowed the identification of nine missense mutations at diagnosis affecting the coding sequence of AMPD3, SUCNR1, FANCD2, INCENP, BSPRY, ZWILCH, HEXDC, KIAA2018 and NUDT9 genes. Six of these mutations (FANCD2, INCENP, BSPRY, ZWILCH, HEXDC, NUDT9) were also detected in the Ph^+ clone re-emerged at the time of disease progression, together with six additional missense mutations affecting the coding sequence of IDH2, DECR1, C4Orf14, MRM1, PRKD2 and TCHP genes. Mutations of isocitrate dehydrogenase 1 and 2 enzyme isoforms (IDH1, IDH2), including the same R140Q found in our study, have recently been reported in in some *de novo* acute myeloid leukemias and in patients with leukemia evolved from a Philadelphia-negative (Ph-) chronic myeloproliferative disease, suggesting that they can be associated with leukemic progression to acute phase both in Ph- and in Ph+ neoplasms. AMPD3 (encoding adenosine monophosphate deaminase 3) and KIAA2018 (encoding a protein with predicted DNA binding and transcriptional regulation activity) genes were found to harbour the same point mutations in 1 out of the 3 additional high Sokal risk patients analyzed. Moreover, the same MRM1 (mitochondrial rRNA methyltransferase 1) mutation detected at the time of progression was found in 3/10 additional BC patient patients analyzed. Digital gene expression profiling and chimeric or alternatively spliced transcripts will also be presented. *Summary*. Our preliminary data highlighted putative key genes whose deregulation may be recurrent in a subset of CML patients and may be linked to disease pathogenesis or progression. Further investigations in a larger series of patients are ongoing.

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0583

HIGH-RESOLUTION GENOME-WIDE ANALYSIS OF COPY NUMBER ALTERATIONS/LOSS OF HETEROZIGOSITY IN CHRONIC MYELOID LEUKEMIA SHOWS THAT HIGH SOKAL RISK PATIENTS HAVE MULTIPLE LOSSES TARGETING DNA REPAIR GENES

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Background. Chronic myeloid leukemia (CML) patients (pts) display a certain degree of clinical heterogeneity that is documented by the varying levels of response to tyrosine kinase inhibitors and is best reflected by the Sokal risk score. Clinical differences must reflect a biological heterogeneity the bases of which are still poorly understood. *Aims.* To perform a genome-wide scan of newly diagnosed CML pts looking for submicroscopic genetic alterations and uniparental disomy targeting key cellular genes. *Methods.* We have used Human 6.0 SNP Arrays (Affymetrix) to perform high-resolution (<1 kb) karyotyping of DNA samples from 73 newly diagnosed chronic phase CML pts. *Results.* Of 189 genes known to be implicated in the cellular DNA repair pathways, 135 (71%) were found to map in regions affected by CNAs or copy-neutral LOH (uniparental disomy, UPD) in 44/73 (60%) pts. However, this was markedly more frequent in high and intermediate Sokal risk pts (20/27, 74% and 16/23, 69%, respectively) than in low Sokal risk pts (8/23, 33%), although neither the total number of detected regions of CNAs/UPD per sample nor the quality control parameters differed significantly across different risk categories. Regions of CNA involving DNA repair genes ranged from 105 Kb to 1.1 Mb and were either focal lesions involving a part or the whole single gene (17% of cases), or more extensive losses/gains including 2 to 84 genes. Monoallelic deletions were much more frequent than amplifications. Regions of UPD involving DNA repair genes were much larger and ranged from 980 kb to 32 Mb. The pathways and genes most frequently affected by CNAs or UPD were: a) Base Excision Repair: MUTYH (loss or UPD, 10 pts); PNKP (loss, 10 pts); NEIL1 (loss, 7 pts); b) Mismatch Repair: PMS2L5 (loss or UPD, 11 pts), MSH2 (loss, 8 pts), POLD1 (loss, 8 pts); c) Nucleotide Excision Repair: ERCC1 (loss, 11 pts), ERCC2 (loss, 10 pts), XAB2 (loss, 9 pts); d) Homologous Recombination: RAD51C (loss or UPD, 7 pts), RAD52 (loss, 7 pts); e) Non-Homologous End-Joining: DCLRE1C (Artemis; loss, 6 pts); REV7 (gain, 6 pts). For some genes (e.g., RAD52), the monoallelic deletion was found to translate into reduced mRNA expression, observation that was also independently confirmed in an additional group of high/intermediate versus low Sokal risk pts. In all the 44 pts, multiple pathways and multiple genes within the same pathway were affected, supporting the hypothesis that the lesions we detected might actually have consequences on DNA integrity despite the known partial functional redundancy of pathways and effectors. *Summary.* We have identified a series of alterations that might result in a perturbation of DNA repair systems in high Sokal risk CML pts. For many of the genes identified in this screen, activating or inactivating mutations are known to occur, and together with overexpression or haploinsufficiency, have been linked to a mutator phenotype in several malignant conditions. We are currently investigating whether this may be the case also in CML. Supported by European LeukemiaNet, AIL, AIRC, PRIN, Fondazione del Monte di Bologna e Ravenna.

0584

DISSECTING MOLECULAR BASES OF HIGH SOKAL VERSUS LOW SOKAL RISK IN CHRONIC MYELOID LEUKEMIA PATIENTS BY GENE EXPRESSION PROFILES OF CD34+ CELLS AT DIAGNOSIS

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Background. CML is a clonal myeloproliferative disease which typically presents in chronic phase (CP), in which malignant progenitor cells proliferate rapidly, still retaining their ability to differentiate, with the disease later evolving to accelerated phase/blast crisis. Even after the introduction of imatinib, the calculation of the Sokal and the Euro prognostic scores has remained essential in clinical practice, since allow to stratify CML patients at different evolutive risk at diagnosis, guiding therapeutic decisions. More recently, numerous research efforts, (which use high-throughput molecular approaches) are ongoing to gain a better understanding about the intrinsic heterogeneity of CML. *Aim.* Here we present data obtained from gene expression profiling (GEP) experiments aimed at the identification of genes and pathways able to discriminate between high vs. low Sokal risk patients at diagnosis and predict the disease course of CP-CML pts at the onset of the disease. *Patients and Methods.* Highly enriched $CD34^+$ cells from peripheral blood were obtained from 107 patients with untreated CML in CP and enrolled in GIMEMA CML WP protocols. Overall, 34 patients were included in the GEP analysis. GEP was performed using the Affymetrix HG-U133 Plus 2.0 platform. Raw data was normalized using the RMA algorithm and filtered. Genes associated with Sokal risk score were selected by a moderated t-statistic (Limma package, P threshold=0.05). *Results.* We showed that 576 genes were differentially expressed between high vs. low Sokal risk patients (P=0.05). Clustering of their expression profiles showed an homogeneous pattern in high Sokal risk patients, where up-regulated genes are mainly related to the glutathione metabolism, the "intrinsic"Wnt signaling (e.g. WNT6, 4E-BP1, ADCY9) and the response to hypoxia and oxidative stress (e.g. GSMT3, GSTT1, GPX1), whereas down-regulated genes are mainly involved in the negative regulation of "extrinsic"pathway of Wnt signaling (e.g. RBX1ligase, Tcf, CSKN1A and TCF7L2). In the second part of the study, the most significant genes were validated by Real-time PCR in a test set of CD34+ cell fraction obtained from 73 newly diagnosed patients. Moreover, the expression of the set of genes related to high or low Sokal risk score has been also evaluated on peripheral blood samples obtained from a different cohort of 100 newly diagnosed CML patients (30 high and 70 low Sokal risk), thus showing a similar pattern of expression of these genes. *Conclusions.* Overall, our data suggests that the expression at diagnosis of a particular array of genes might drive the evolutive risk of CML patients.

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0585

SEVERE TELOMERIC LOSS OCCURS IN PH-NEGATIVE HEMATOPOIESIS EMERGING AFTER SUCCESSFUL TREATMENT OF CHRONIC MYELOID LEUKEMIA AND ASSOCIATES WITH ACQUIRED CYTOGENETIC LESIONS AND LOW HEMOGLOBIN LEVELS

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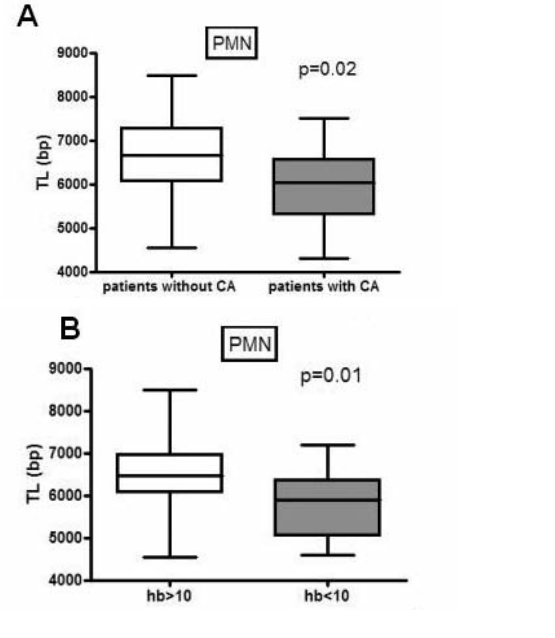
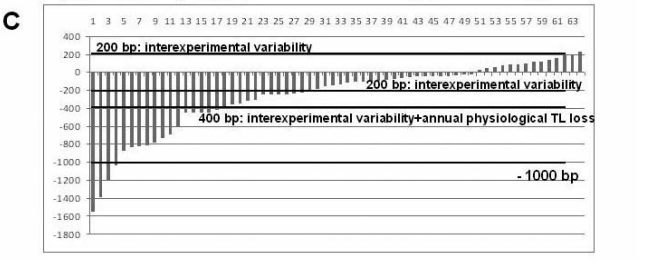
Background and Aims. Telomere attrition represents a useful marker of proliferative and oxidative stress and provide useful insights to monitor the genetic integrity of the hematopoietic compartment. This approach has been used to assess the genetic integrity of Ph-negative hematopoietic cells (HCs) repopulating the bone marrow after successful chronic myeloid leukemia (CML) treatment.





Patients and Methods. We investigated 81 CML patients with persistent (≥12 months) complete cytogenetic remission (CCR). Median age was 62 (23-88), M/F ratio was 1.5. Median time from diagnosis and CCR were 54 months (25-217) and 37 months (12-191) respectively. Fifteen patients had acquired cytogenetic abnormalities (CA) (del7: 4 patients, +8: 5 patients, del5q: 2 patients, del or +Y: 2 patients, other CA 2 patients). Telomere length (TL) analysis was performed by Southern Blotting on polymorphonucleates (PMN) and on monocyte-depleted PBMC (MD-PBMC) to monitor the myeloid and lymphoid compartments. Two age-matched control groups were used: a) 109 healthy subjects; b) 20 patients receiving imatinib for a gastrointestinal stromal tumor (GIST) (to investigate treatment-related effects on TL). Prospective follow-up monitoring of TL was performed on 64 patients (median time 11 months, range 6-20). *Results.* PMN (but not MD-PBMC) from CML patients showed a major erosion of their telomeric DNA (median loss 1294 bp P<0.001). Telomere attrition appeared to be CML-specific as GIST patients had a TL not significantly different from healthy subjects with a median TL of 7152 (P=1). A multivariate general linear model (GLM) was applied on CML patients and controls: CML and age were predictors of telomeric attrition (both P<0.001), while sex and GIST history had no significant impact (P=0.6 and 0.8 respectively). We then analyzed whether clinical and biological features of CML patients were predictive of telomere attrition. We found no association with sex, Sokal score, molecular remission or treatment schedule. Of note, we found increased telomere attrition and acquired CA compared to CML patients without CA (P<0.030, Figure 1A). When a multivariate model was applied to the CML series only age and CA were predictors of telomeric damage (P=0.013 and 0.001 respectively). When telomere shortening was correlated to hematopoietic function we found no association with WBC or platelets level. However we found an association between telomere shortening and severe anemia (Hb<10 gr/dL, P=0.003) (Figure 1B). TL was substantially stable overtime in the majority of patients. In none of the patients TL recovery overtime was observed. However in 16 (25%) patients a non-physiological telomeric loss was noticed (Figure 1C). *Conclusions.* I) Ph-negative HCs display severe telomeric loss, compared to healthy controls and CML-free subjects treated with imatinib; II) telomere erosion is more pronounced in patients with CA; III) an association between short telomeres and anemia was observed; IV) telomere loss is persistent and potentially worsening in a subset of cases. Our results suggest that Ph-negative hematopoiesis in CML patients has undergone significant genetic damage and warrants careful clinical monitoring.

Red cell disorders

0586

UBIQUITINATION OF THE ERYTHROPOIETIN RECEPTOR IS NOT REQUIRED FOR EPO-INDUCED INTERNALIZATION BUT IS IMPORTANT FOR ENDO-LYSOSOMAL SORTING AND DOWN-REGULATION

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Background. Erythropoietin (Epo) is the primary cytokine controlling red blood cell production and its function is mediated through the Epo receptor (EpoR). Epo binding to the EpoR initiates signaling cascades that result in the survival, proliferation, and differentiation of erythroid progenitor cells. Following Epo binding, cell surface EpoRs are rapidly internalized and are subsequently degraded intracellularly. Down-regulation of the EpoR critically controls the amplitude and duration of Epo signaling. It was shown that EpoR is ubiquitinated upon Epo stimulation and the E3 ubiquitin ligase β-TrCP (β-transducin repeat-containing protein) inducibly binds the EpoR cytoplasmic domain upon stimulation. Mutation of the predicted β-TrCP recognition motif in the EpoR abolished β-TrCP binding and blocked EpoR ubiquitination and degradation. However, the exact process(es) in receptor down-regulation controlled by ubiquitination are not entirely understood. *Aims*. We aimed to examine the role of EpoR ubiquitination in receptor internalization, endosomal-lysosomal targeting and degradation. *Methods and Results*. We engineered a mutant receptor that is unable to be ubiquitinated, in which all five EpoR cytosolic lysines were replaced with arginines. Although this lysineless receptor (AllKR) was not ubiquitinated upon Epo stimulation, it internalized similarly to wild-type EpoR. In addition, fusion of (mono)ubiquitin to the wild-type EpoR cytoplasmic domain did not trigger receptor internalization. Therefore, EpoR ubiquitination is not necessary or sufficient for ligand-induced EpoR internalization. The fate of EpoRs upon Epo induction was examined by confocal immuno-fluorescence microscopy. 25 min post Epo treatment, HA-tagged wild-type EpoRs shifted from the plasma membrane to an internal compartment that co-localizes with the early endosomal marker, EEA1 (early endosome antigen 1). At 35 min post Epo stimulation, EpoR co-localized with the lysosomal-associated membrane protein 2 (LAMP2). In contrast, HA-tagged AllKRs co-localized with EEA1 25 min post Epo stimulation but were not efficiently sorted to the lysosome, as few receptors co-localized with LAMP2 35 min post Epo treatment. Consistently, Epo-induced degradation of AllKR was dramatically impaired. Therefore, EpoR ubiquitination is not required for internalization but is important for endo-lysosomal sorting and down-regulation. To identify the EpoR lysine residue(s) that are ubiquitinated upon Epo stimulation, we examined mutated receptors in which individual lysine was replaced with arginine. Ligand-induced degradation was defective in K428R, while other lysine mutants behaved normally. Importantly, receptor ubiquitination and normal degradation were restored in cells where K428 was re-established on the lysineless receptor, indicating that K428 may be the major target of ubiquitination. *Conclusion.* We show in this work that ubiquitination of the EpoR itself is not required for internalization but is important for endo-lysosomal sorting and receptor down-regulation upon stimulation.

0587

A NOVEL APPROACH TO CELL-TARGETED COMPLEMENT INHIBITION IN PNH: THE HUMAN COMPLEMENT RECEPTOR 2/FACTOR H FUSION PROTEIN TT30 PREVENTS BOTH HEMOLYSIS AND UPSTREAM C3-OPSONIZATION OF PNH ERYTHROCYTES

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Background. In paroxysmal nocturnal hemoglobinuria (PNH) severe hemolytic anemia results from the fact that spontaneous complement alternative pathway (CAP)-activation fails to be controlled, because affected RBCs are deficient in CD55 and CD59. Eculizumab has proven highly effective in controlling MAC-mediated intravascular hemolysis (IVH); however, we have recently reported that its hematological benefit may be limited by the emergence of C3-mediated extravascular hemolysis (EVH). TT30 is a recombinant human fusion protein consisting of (a) the iC3b/C3d-binding region of C receptor 2 (CR2) and (b) the inhibitory domain of the CAP regulator factor H (fH). The resulting molecule is a novel CAP inhibitor, based on the notion that the CR2 component will localize the molecule specifically to target cells on which C-activation is taking place; whereas the fH component will help to control CAP activity.*Aims.* To investigate whether TT30 can protect PNH RBCs from hemolysis and can prevent their C3-fragments (C3frag)-opsonization after C-activation. *Methods.* We have incubated washed RBCs from PNH patients with ABO-matched sera from healthy volunteers, and activated the CAP by acidification. These conditions allow us to monitor not only the terminal C pathway responsible for hemolysis, but also the early C-activation responsible for C3frag-opsonization of RBCs. Both hemolysis and RBC C3frag-opsonization were serially measured by flow-cytometry. *Results.* We have studied RBCs from 9 untreated and 4 PNH patients on eculizumab. After 24h incubation in acidified normal serum, 74±16% of PNH RBCs from untreated patients were lysed; C-mediated hemolysis was confirmed by the presence of C3frag on RBC ghosts. Addition of TT30 produced a concentration-dependent inhibition of C mediated hemolysis; with 1 μM (65 μg/mL) TT30 hemolysis was reduced to 14±26% and with 3 μM TT30 it was reduced to 5±7%. Equimolar concentrations of human fH produced much less inhibition of hemolysis (about 50% lysis), supporting the notion that TT30 is cell-targeted. Of note, unlike with exposure to eculizumab, *in vivo* or *in vitro* (Sica, EHA 2010), surviving PNH RBCs did not show any C3frag-deposition on their surface. When incubated with ABO-matched normal sera, lysis of PNH RBCs from patients on eculizumab was comparable to that of RBCs from untreated patients; with 3 μM TT30 hemolysis was 31±8%. This reduced protection from hemolysis of RBCs from PNH patients on eculizumab could be attributed to increased susceptibility to lysis of C3frag+ RBCs. However, RBCs from treated patients that had survived 24h incubation in acidified serum with TT30 were protected from lysis even after serum removal and re-exposure to fresh acidified sera, for up to 24h: i.e., longer than those from untreated patients. This in turn could be explained by the fact that C3frag pre-loading *in vivo* is helping to target TT30, when added *in vitro*, to the RBC surface. *Conclusions*. Our experiments support the concept that TT30 targets C3frag on PNH RBCs to deliver surface-based fH activity: thus, by blocking the CAP and its amplification loop, TT30 inhibits both hemolysis and C3frag-opsonization of PNH RBCs. In principle, TT30 is a candidate agent to control both MAC-mediated IVH and C3-mediated EVH in patients with PNH.

0588

THE BRAZILIAN HEREDITARY PERSISTENCE OF FETAL HEMOGLOBIN TYPE MUTATION REDUCES THE BINDING OF THE NF-E1/YY1 TRANSCRIPTION FACTOR AND MAY BE RESPONSIBLE FOR THE REACTIVATION OF THE GAMMA GLOBIN GENE

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Non-deletional hereditary persistence of fetal hemoglobin (nHPFH) results in the continued expression of the gamma globin gene and production of hemoglobin (Hb) F in adults. High levels of HbF can ameliorate several manifestations of sickle cell disease and beta-thalassemia symptoms. Understanding the molecular mechanisms leading to reactivation or repression of gamma globin gene expression will lead to the development of new therapies to treat these patients. The Brazilian nHPFH type was identified in 1990 as a C→G mutation at position -195 of the A gamma globin promoter. In order to elucidate the mechanism mainly responsible for the Brazilian nHPFH phenotype, some studies have been carried out, but this mechanism remains unclear. The aim of this study was to identify transcription factors (TF) involved in the reactivation or repression of the A gamma globin gene in the Brazilian nHPFH type. Nuclear extracts from day 10 of primary human erythroblast cultures of Brazilian nHPFH type subjects and control subjects (C) were used to profile the activities of TFs using Protein-DNA Array technology. Using this array method, more than 20 TFs, whose DNA binding activities were modulated by the Brazilian nHPFH type, were identified. These TFs include NF-E1/YY1, HOX, Tat, Pax-1, TEF1, HIF-1 and HFH-3. To verify the array data with a secondary assay, nuclear extracts were used to conduct electrophoretic mobility shift assay (EMSA) with some TFs: NF-E1/YY1, Tat, HOX and TEF-1. The EMSA analysis validated the previous array *Results.* Interestingly, NF-E1/YY1, a transcription factor that represses epsilon and gamma globin, showed decreased activity in nuclear extracts of Brazilian nHPFH type subjects in the two methods used - DNA-protein array and EMSA. The consensus sites for NF-

E1/YY1 binding from various promoters have been found to share a CCAN core motif. The C→G mutation at -195 may disrupt this motif and causes decreased NF-E1/YY1 binding. This observation suggests that the -195 C→G substitution may abrogate NF-E1/YY1 in this promoter region, probably resulting in the reactivation of the A gamma globin gene. These results provide the first *in vitro* evidence for the probably molecular mechanism of reactivation of the A gamma globin gene in Brazilian type nHPFH.

0589

OUTCOMES OF UNRELATED CORD BLOOD TRANSPLANT IN PATIENTS WITH HEMOGLOBINOPATHIES

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Allogeneic hematopoietic cell transplantation (HCT) from HLA-identical donors is curative in patients with hemoglobinopathies. In order to extend the availability of HCT, unrelated cord blood transplantation (UCBT) has been investigated. Between 1996 and 2009, 51 patients receiving a single UCBT for hemoglobinopathy were reported to the Eurocord and CIBMTR registries. Thirty-five had thalassemia major (Thal) and 16 had sickle cell disease (SCD). All Thal patients were transfusion-dependent with a median time from diagnosis to UCBT of 26 months. Twelve of 16 patients with SCD had a history of stroke or central nervous system involvment. Median age at UCBT was 3.9 years and 6.3 years for Thal and SCD, respectively, and median follow-up was 2 years. Cord units were matched at 6 of 6 (15%), 5 of 6 (34%) and 4 of 6 (51%) HLA loci (antigen-level for class 1, allele-level for class 2). Median infused cell dose was 4.9×10^7/kg (1.5-13). Forty patients received a myeloablative conditioning regimen (MAC) with busulfan (BU) and cyclophosphamide (n=33) or BU with other agents (n=6). Reduced intensity conditioning regimens (RIC) (n=10) were fludarabine-based with BU<8 mg/kg or melphalan<150 mg/m^2. Cumulative incidence (CI) of neutrophil recovery at 60 days was 72±6%, with 37 of 51 patients reaching neutrophil recovery at a median time of 22 days. UCBT performed after 2004 (84% vs 68%, P=0.005) were associated with improved neutrophil recovery. According to conditioning regimen used, 29 of 40 who received a MAC and 7 of 10 who had a RIC achieved neutrophil recovery. Day 100 chimerism analysis was available for 48 patients: 20 patients had complete donor chimerism, 4 mixed chimerism and 24 autologous recovery. Among 14 patients who did not achieve neutrophil recovery, 5 had available data on subsequent treatment. Two received an autologous back-up and one received a second UCBT which engrafted. CI of grade II-IV acute-graft versus-host disease (GVHD) was 23% and 9 patients had chronic GVHD (2 with extensive disease). The 2-year probability of overall survival was 72%. Thirty-eight patients (23 Thal, 15 SCD) are alive, 15 with full donor chimerism (8 Thal, 7 SCD). Of the 13 deaths, 5 occurred prior to day-100, mainly due to infections. No deaths due to GVHD were reported. Despite the small number of subjects, these results show a particularly high risk of graft failure after UCBT for hemoglobinopathy. Novel approaches modifying the conditioning regimen and/or increasing cell dose in prospective clinical trials are needed.

0590

THE ROLE OF VLA-4 AND CX3CR1 ON RECRUITMENT OF T CELLS INTO BONE MARROW IN PATIENTS WITH APLASTIC ANEMIA

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Background. Aplastic anemia (AA) is a typical example of the human bone marrow failure syndromes. More and more evidence has proved acquired AA is a T-cell-mediated autoimmune disease. Peripheral blood cytopenia and bone marrow hypoplasia due to T-cell-mediated destruction and apoptosis of hematopoietic stem and progenitor cells is considered to be the essential pathogenesis of acquired AA. However, the transportation of T-cells into bone marrow has intimate relationship with the expression of adhesion molecules and chemokine receptors on T-cells. Recently, Bob Olsson and coworkers reported that elevated expression of VLA-4 and CX3CR1 was involved in recruitment of T-cells into bone marrow in patients with idiopathic thrombocytopenic purpura (ITP), which intiated us to investigate the expression of VLA-4 and CX3CR1 on T cells in acquired AA patients. *Aims.* This study was to analyze the role of VLA-4 and CX3CR1 on recruitment of T-cells into bone marrow in patients with acquired AA and povide new evidence for the immunologic mechanism of acquired AA. *Methods.* The expression of VLA-4 and CX3CR1 on T cells from peripheral blood and bone marrow was analyzed by flow cytometry in 27 AA patients (15 with severe aplastic anemia and 12 with moderate aplastic anemia) and 21 controls. Confocal scanning microscopic analysis of immunoflourescence was introduced to detect the expression of VLA-4 and CX3CR1 in paraffin-embedded bone marrow biopsies from 17 AA patients and 10 controls. *Results.* Both in peripheral blood and bone marrow, there was no statistically significant difference in the mean fluorescence intensity (MFI) or the percentage of VLA-4 on T-cells ($CD3^+$ T-cells, $CD3^+/CD4^+$ T-cells and $CD3^+/CD8^+$ T-cells) between acquired AA patients and controls. However, the percentages of $CX3CR1^+/CD3^+$ cells and $CX3CR1^+/CD3^+/CD8^+$ cells from peripheral blood as well as bone marrow were increased significantly in acquired AA patients compared with controls. This finding was confirmed by confocal scanning microscopic analysis of immunoflourescence of BM biopsies. *Conclusions.* Acquired AA, as an immune-mediated disease, is inseparable with accumulation and activation of oligoclonally expanded cytotoxic T-cells in bone marrow. Increased expression of certain adhesion molecules or chemokine receptors may contribute to the trafficking of T-cells from peripheral blood to bone marrow, and CX3CR1 is an important one of these molecules involved in T-cell homing in acquired AA.

Genomics

0591

THE ROLE OF MEIS1 IN PRIMITIVE AND DEFINITIVE HAEMATOPOIESIS AND VASCULAR PATTERNING DURING ZEBRAFISH DEVELOPMENT

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Background. Meis homeobox 1 (Meis1) is a three-amino-acid loop extension (TALE) homeodomain protein that was first identified as a common viral integration site in myeloid leukaemic cells of BXH-2 mice. It is considered as a critical effector, possibly downstream of Scl, that has a rate-limiting regulatory role in mixed lineage leukaemia (MLL) and limb development. In addition to its role in leukaemogenesis, several lines of evidence indicate a key role of meis1 in normal haematopoiesis. Along with *hoxa-9* and *pbx1* genes, *meis1* is transcribed in human CD34$^+$ haematopoietic stem cells (HSCs) and is down regulated following differentiation, except in the megakaryocytic lineage where transcript levels remain abundant. Mice lacking *meis1* are not viable and die early during development (E11.5-14.5) due to lack of megakaryocytes and extensive haemorrhaging, and while erythromyeloid lineages are present the number of colony-forming cells is severely reduced. Mutant fetal liver cells also fail to radioprotect lethally irradiated animals and they compete poorly in repopulation assays. In addition, *meis1* mutant mice display localised defects in vascular patterning with capillary network being mainly affected. *Aims.* Though much is known about the role of *meis1* in leukaemogenesis, its function in normal haematopoiesis, remains largely unclear. Here we characterise the role of the proto-oncogene, *meis1*, during zebrafish primitive and definitive haematopoiesis and vascular patterning. *Methods and Results.* Using antisense morpholino oligonucleotides (MO) to interrupt *meis1* expression we find that, although primitive macrophage development can occur unhampered, the posterior erythroid differentiation requires *meis1*, resulting in severe decrease in number of mature erythrocytes. Definitive haematopoiesis was also critically dependent on functional *meis1* signalling. MO injected embryos showed normal specification of haematopoietic stem cells (HSCs) mediated by c-myb expression. They failed, however, to seed the caudal haematopoietic tissue (CHT) and differentiate into the mature progeny, ultimately producing significantly fewer myeloid cells and completely lacking lymphoid cells and thrombocytes. In addition, *meis1* MO knockdown leads to dramatic single arteriovenous tube formation and impairment of caudal vein plexus development. In *meis1* MO injected embryos we observed a dramatic decrease in the expression of the arterial marker Ephrinb2a (Ephb2) and a concurrent ectopic expression of the venous marker *flt4*. From these observations we inferred that the loss of *meis1* function leads to a major disruption of the segregation of angioblasts and the associated vessels lumen formation. We also find that knockdown of *pbx1* results in a strikingly similar phenotype to that of *meis1* knockdown. *Summary.* Thus, these results implicate that meis1, jointly with pbx1, regulates both primitive and definitive haematopoiesis as well as vascular development.

0592

C/EBPa INSTRUCTS EARLY THYMIC PROGENITORS TOWARDS CELLS WITH MYELOID AND DENDRITIC CELL POTENTIAL

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Background. In contrast to the classic scheme of haematopoietic cell differentiation, which proposes a symmetrical early branch into lymphoid and myeloid lineages in the bone marrow, myeloid potential has recently been shown to be retained among some thymic progenitor cells. Within the progenitor compartment myeloid potential was predominantly confined to the earliest stage of T cell development, the DN1 (double-negative for CD4/CD8 and c-kit$^+$CD25$^-$) population, reduced in DN2 (c-kit$^+$CD25$^+$) and absent in DN3 (c-kit-CD25$^+$) cells. However, mechanisms responsible for retained myeloid potential as well as markers for such progenitors are not known. *Methods and Aims.* To delineate a role of C/EBPα, which is a crucial transcription factor in myeloid cell development and indispensable for granulocyte formation, in early thymic progenitors with retained myeloid potential, we used a recently developed mouse model expressing Cre recombinase under control of the endogenous *Cebpa* promoter and an inducible EYFP allele (Cebpa^{cre}/+ R26 EYFP mice). This model enabled us to track single cells that either express Cebpa or are the progeny of Cebpa-expressing cells and to evaluate their developmental potential. *Results.* The highest fraction of Cebpa/EYFP$^+$ cells was found within the DN1 population (22%), whereas the percentage of Cebpa/EYFP$^+$ cells gradually decreased during T cell differentiation resulting in the lowest fraction among the most mature single positive CD3$^+$ thymocytes (9%) suggesting either a proliferation disadvantage of Cebpa/EYFP$^+$ progenitor cells during T cell development or an alternative cellular fate of Cebpa/EYFP$^+$ progenitors. Indeed, Cebpa/EYFP$^+$ DN1/2 cells contained approximately 7 times higher numbers of myeloid colony-forming cells relative to EYFP- DN1/2 cells. Since thymic dendritic cells (DC) have been proposed to descend from DN1/2 cells, we also analyzed the distribution of Cebpa/EYFP in this cell population. About 20% of CD11cintB220$^+$ plasmacytoid DC expressed EYFP, whereas 50% of CD11chighB220$^-$ conventional DC were Cebpa/EYFP$^+$. Because Cebpa is not expressed during later stages of DC differentiation, these data imply that significant fractions of conventional and, to a lesser extent, plasmacytoid DC in the thymus are derived from Cebpa-expressing progenitors. Accordingly, sorted Cebpa/EYFP$^+$ DN1/2 cells displayed a higher potential to form dendritic cells *in vitro* as compared to EYFP$^-$ cells. Given the loss of Cebpa/EYFP$^+$ cells during T cell development *in vivo*, we then asked whether C/EBPα affects the expansion of DN1/2 cells in a T cell inducing environment. EYFP$^+$ and EYFP- DN1/2 cells expanded at comparable proliferation rates in fetal thymic organ cultures (FTOC), a result supported by competitive repopulation experiments. However, a significantly higher proportion of cells obtained from EYFP$^+$ DN1/2 cells remained double-negative (CD4$^-$CD8$^-$) after 3 weeks of culture, relative to the EYFP- DN1/2. These results suggest that although their ability to proliferate in FTOC is not affected, Cebpa/EYFP$^+$ DN1/2 cells are hampered in differentiation towards CD4$^+$CD8$^+$ thymocytes. *Conclusions.* Myeloid potential in DN1/2 progenitors is predominantly confined to cells, which express Cebpa or are the progeny of Cebpa-expressing cells. C/EBPα instructs these cells to develop toward clonogenic myeloid cells or DC and thus represents a marker for myeloid potential in early thymic progenitors.

0593

CEBPA DOUBLE MUTATIONS IMPACT FAVORABLY ON THE OUTCOME OF OLDER ADULTS WITH WILD-TYPE NPM1 CYTOGENETICALLY NORMAL ACUTE MYELOID LEUKEMIA AND ARE ASSOCIATED WITH DISTINCT GENE AND MICRORNA EXPRESSION

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Background. Mutations in *CEBPA*, a gene essential for myeloid differentiation, have been associated with a favorable prognosis in cytogenetically normal acute myeloid leukemia (CN-AML). Recent reports found this favorable impact restricted to double-mutated *CEBPA* (*CEBPA*-2mut), while single-mutated *CEBPA* (*CEBPA*-1mut) associated with a prognosis similar to that of *CEBPA* wild-type (*CEBPA*-wt). None of these studies focused on the clinical significance of *CEBPA* mutations in older [≥60 years (y)] CN-AML patients who generally have a poor outcome. *Aims.* To analyze the clinical impact of *CEBPA* mutations in the context of other molecular markers in older *de novo* CN-AML and provide biologic insights through gene- and microRNA-expression profiling. *Methods.* Pretherapy marrow or blood from 243 patients (60-83y) treated intensively on front-line cytarabine/daunorubicin-based CALGB protocols were analyzed centrally for *CEBPA* mutations and other molecular markers [*NPM1*, FLT3 (ie, *FLT3*-ITD, *FLT3*-TKD), *WT1, MLL, IDH1* and *IDH2* mutations and BAALC, ERG and MN1 expression]. Allelic discrimination of *CEBPA*-2mut was assessed by cloning the entire *CEBPA* coding sequence. Gene and microRNA expression were profiled by Affymetrix U133 plus 2.0 and OSU_CCC v4.0 arrays, respectively. *Results.* Twenty-nine (12%) patients harbored *CEBPA* mutations, including 9 with *CEBPA*-2mut, all of whom had bi-allelic *CEBPA* mutations and

were NPM1 wild-type (*NPM1*-wt). Compared with *CEBPA*-wt and *CEBPA*-1mut patients, *CEBPA*-2mut patients had lower platelet counts (P=.015 and P=.009, respectively) and were more often NPM1-wt (P=.003 and P=.045, respectively). No significant differences were observed for complete remission rates, disease-free survival (DFS) or overall survival (OS) among *CEBPA*-2mut, *CEBPA*-1mut, and *CEBPA*-wt patients. When restricting analyses to NPM1-wt patients (n=103), a large molecular subset of older CN-AML we previously reported to have a particularly poor prognosis, we still observed no outcome differences between *CEBPA*-1mut and *CEBPA*-wt patients. However, *CEBPA*-2mut patients tended to have a longer DFS (P=.06; 3y-rates 43% v 4%) and a significantly longer OS (P=.03; 3y-rates 33% v 6%) compared with the combined group of *CEBPA*-1mut (n=11) and *CEBPA*-wt patients (n=83). Expression profiling distinguished *CEBPA*-2mut from *CEBPA*-wt patients by a signature comprising 307 differentially expressed genes. This signature predicted *CEBPA*-2mut status with 99.5% cross-validated accuracy. Prominent signature features were up-regulation of *CEBPA* and the hematopoietic progenitor markers CD34, CD38, CD7 and CD96, and down-regulation of the leukemia-associated RUNX1, HOXA and HOXB genes. *CEBPA*-2mut could also be distinguished from *CEBPA*-1mut patients by a signature comprising 173 genes; as in the comparison with *CEBPA*-wt patients, CD7 was up-regulated and *RUNX1, HOXA,* and *HOXB* down-regulated in *CEBPA*-2mut patients. Based on this signature, *CEBPA*-2mut status was predicted with 85.7% cross-validated accuracy. Consistent with the HOX down-regulation, *CEBPA*-2mut compared to *CEBPA*-wt and to *CEBPA*-1mut patients had significant down-regulation of miR-10a, a microRNA embedded in the HOXB gene cluster. *CEBPA*-1mut patients were not as readily distinguishable from *CEBPA*-wt patients, with only 98 differentially expressed genes and cross-validated prediction accuracy of only 64.3% for *CEBPA*-1mut patients. *Conclusions.* In older *de novo* CN-AML, *CEBPA*-2mut associates with more favorable outcome within the prognostically adverse NPM1-wt subset. The gene and microRNA expression characteristics suggest that CEBPA-2mut represents a distinct genetic entity within CN-AML.

0594

MICRORNA NETWORKS IN LEUKEMIA

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Background and *Aims.* MicroRNAs (miRNAs) are small non-coding RNAs whose expression is de-regulated in human leukemias. Typically miRNAs have been studied by using the gene profiling approach: i.e. each miRNA has been studied for its single contribution to differential expression or, at the most, to a compact predictive signature. Here, we propose a paradigm shift to the study of miRNAs role in cancer by applying a "whole cell"systems biology approach. We have performed detailed analysis of coordinated miRNA activities and built miRNA gene networks by using large expression miRNA datasets obtained from chronic lymphocytic leukemias (CLL) and acute myeloid leukemia (AML) patients. *Methods.* The miRNA expression datasets were obtained using the OSU-CCC custom miRNA microarray version 2, 3 and 4 and all, except 326 AML cases were already used in publications by our group and deposited in public microarray databases. The microarray experiments, data normalization (quantiles) and filters used were performed as previously described in the papers cited below. Banjo was used for inferring the bayesian network and the MCL clustering algorhytm was used to extract (and visualize) coherent groups of nodes from the expression network. 'Hub"gene of an expression module was defined as a gene with strong intramodule connectivity, as measured by the sum of its pair-wise weighted correlation to all other genes in the same module. *Results.* First, we generated a single network reflecting the miRNome of AML. We assayed mature miRNAs in 598 AML samples (480 newly diagnosed and 109 relapsed patients). Cytogenetics and molecular information was available for 560 patients and follows this distribution: complex karyotype: 136 (24%), t(8;21) and inv16:12(2%), t(15;17):+8(1.4%), t(11q23):14(2.5%), other abnormality: 96(17%), cytogenetically normal: 302(53%), including FLT3-ITD(42/189), NPM1-mutation (103/220). Results from this analysis revealed a well connected network, except two miRNA families; miR-181 and miR-146 that were positioned in a separate cluster. Both miRNA families have been associated with NFκB (Marcucci G,et al NEJM 2008). On the main cluster, two miRNAs with known roles in myeloid leukemias; miR-328 and miR-29b acted as a hub in the AML. The oncogene miR-155 was located on the main cluster and in close association with miR-223, miR-92a, miR-25 and miR-32. Next, using the same approach described in AML, we assayed mature miRNAs in 254 CLL patients from 3 different datasets (Calin G, et al NEJM 2005; Calin G.et al Cancer Cell 2007 and Visone R,et al Blood 2009). In CLL we observed 3 separated clusters. The main cluster was dominated by miR-21 which acts as a hub. There was a direct association between miR-21 and the oncogenic miR-17-20-106a cluster. While the miR-29 was a hub in AML, it is a branch in CLL. Two isolated clusters were found: miR-23a and -b and the miR-15a/miR-16-1 cluster. *Conclusions.* In summary, the complexity of our expression database enabled us to perform detailed analysis of coordinated miRNA activities. We inferred, for the first time, genetic networks for miRNAs in human leukemias. Intriguingly, some prominent cancer associated miRNAs were separated from the coordinate control of the general transcriptional program.

0595

ABERRATIONS OF MULTIPLE GENES REGULATING NF KAPPA B PATHWAY IN MALIGNANT LYMPHOMA

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Nucleo factor kappa B (NFκB) plays an important role in proliferation and development of lymphocyte. Aberrant activation of NFκB is a characteristic feature of several subsets of B-lineage malignant lymphomas. Previous reports revealed various genomic abnormalities in genes regulating NFκB pathway. Recently, our group showed that A20, a negative regulator of this pathway, is frequently inactivated in B-cell malignant lymphomas. However, the landscape of genomic aberrations in NF kappa B pathway genes has not fully understood. In this study, to clarify the genetic basis of the aberrant NFκB activation, we performed genome-wide analysis of copy number alterations as well as allelic imbalances of primary B-lineage lymphoma specimens using single nucleotide polymorphism genomic microarray. We also searched for possible mutations in multiple genes encoding positive and negative regulators of NFκB pathway, including CARD11, CYLD, IKK family genes and TRAF family genes and IκB family genes. This study included 238 primary lymphoma samples, including 64 samples of diffuse large B-cell lymphomas (DLBCL), 52 of follicular lymphomas (FL), 35 of mantle cell lymphomas (MCL), and 87 of mucosa-associated tissue (MALT) lymphomas. Through a genome-wide analysis, we identified that each histology type had a unique genomic signature, suggesting a distinctive underlying molecular pathogenesis for different histology types. As expected, genomic abnormalities in NFκB pathway associated genes were frequently detected. In total, more than 40% of analyzed samples had genomic abnormalities in genes on NFκB pathway. Fifty cases had loss of heterozygosity at A20 locus, 24 of which had mutation or deletion in the remaining A20 allele. Thirty-nine cases had copy number gains (8 cases of amplification is included) at REL locus, encoding one of NFκB components. Fourteen cases had gains/amplifications at TRAF6, positive regulator of NFκB pathway. In addition, mutations in CARD11 and IκBε were also detected in DLBCL and MALT. To also assess the role of uncontrolled signaling of NFκB pathway in lymphomagenesis, we re-expressed wild-type A20 in two lymphoma-derived cell lines without normal functional A20 alleles (KM-H2 and L1236, derived from Hodgkin lymphoma). In both cells, re-expression of wild-type A20 resulted in suppression of cell growth and induction of apoptosis, accompanied by down-regulation of NFκB activation. According to array-based expression analysis, the list of genes suppressed by A20 expression included various cytokines (ex. M-CSF and various chemokines), receptor for various stimuli (ex. CD40 and CD58) and proteins regulating NF kappa B pathway (ex. NFKB2, Bcl-x and IκBε). In conclusion, our study demonstrated that uncontrolled NFκB signaling caused by alterations of multiple genes is a common feature of B-lineage lymphomas.

Acute lymphoblastic leukemia - Clinical

0596

A PEDIATRIC-INSPIRED INTENSIFIED THERAPY OF ADULTS T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA REVEALS THE FAVORABLE OUTCOME OF NOTCH1/FBXW7 MUTATIONS, BUT NOT OF LOW ERG/BAALC EXPRESSION: A GRAALL STUDY

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T-ALL corresponds to a heterogeneous group of acute leukemia which account for 10% of pediatric and 35% of Ph1-negative adult ALL. Several genetic abnormalities have been identified in T-ALL but therapeutic stratification still relies on clinical markers. The French study group (GRAALL) reported a highly significant improvement of adult T-ALL outcome using a pediatric-inspired intensified therapy, but relapses are still frequently fatal. Therefore identification of molecular risk factors that allow early and effective treatment stratification is needed. NOTCH1 and/or FBXW7 mutations (N/Fmut) lead to activation of NOTCH1 pathway in more than 70% of both pediatric and adult T-ALL and have been reported to be of favorable outcome in pediatric T-ALL but either it is also the case in adult T-ALL remain unclear. Low ERG and BAALC (E/Blow) transcript expression, initially described as of good prognosis in AML, was reported by the German GMALL group to predict also a highly favorable outcome in 41% of adult T-ALL. More recently, they showed that N/Fmut is associated to better EFS within the group of E/Blow patients but is not predictive of a better outcome in the overall cohort of adult T-ALL. Controversial N/Fmut prognosis impact in adult T-ALL is likely due to the small number of patients included in some previously studies and more importantly to the differences in therapy regimens. Herein, we investigated the prognosis impact of N/Fmut and E/Blow in a large cohort of 227 adult T-ALL enrolled in the LALA-94 (n=86) or GRAALL-03/05 (n=141) protocol. The incidence of N/Fmut (159/227=70%) was similar in both protocols. N/Fmut patients when compared to non-mutant, had a better pednisone response (62% vs 36%; P=.005), a higher rate of complete remission (CR) (96% vs 91%;P=.11) and a lower relapse rate (35% vs 53%;P=.02). N/Fmut had a better 3-years EFS (53% vs 37%;P=.002) and OS (61% vs 48%;P=.008). When LALA-94 and GRAALL-03/05 protocols were analyzed separately, N/Fmut retained their higher OS in LALA-94 (48% vs 42%;P=.03) but lost their significant higher EFS (35% vs 31%;P=.11). In contrast, the most recent GRAALL-03/05 trials, highlight the very good prognosis of N/Fmut (OS=72% vs 50%, EFS=69% vs 41%; P<.01). We next quantified E/B transcript expression in 193 patients of whom sufficient RNA was available. 44% (n=84) of patients were classiffied E/Blow as previously reported by Baldus et al. E/Blow patients had higher CR rate (97.5% vs 90.6%;P=.05) ,a trend toward a better EFS (58% vs 47%; P=.12), but not better OS (61%vs 59%; P=.3). E/Blow prognosis impact was further investigated within N/Fmut and non-mutated cases separately. There is no difference in outcome in the non-mutated group according to E/B expression level. In contrast, within N/Fmut , E/Blow demonstrated a trend to a better EFS (P=.19). Taken overall, our data demonstrate that N/Fmut, but not E/Blow, identify a major subgroup (70%) of adult T-ALL with a highly favorable outcome. This benefit is most striking with the pediatric inspired GRAALL-03/05 trials and could justify individual therapeutic stratification for T-ALL.

0597

IKZF1 DELETIONS PREDICT RELAPSE IN UNIFORMLY TREATED PEDIATRIC PRECURSOR B-ALL

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Background. Relapse is the most common cause of treatment failure in pediatric acute lymphoblastic leukemia (ALL) and is difficult to predict in the majority of cases. *Aims.* To explore the prognostic impact of recurrent DNA copy number abnormalities on relapse in children diagnosed with precursor B-ALL. *Methods.* We performed genome-wide copy number profiling of 34 paired diagnosis-relapse samples, and validated the findings in an unselected cohort of 131 precursor B-ALL cases, enrolled in the dexamethasone-based Dutch Childhood Oncology Group (DCOG) treatment protocol ALL9. *Results.* We observed that diagnosis and relapse samples were genomically distinct in the majority of cases. Lesions detected at diagnosis were frequently absent at relapse, including those encompassing recurrent targets in precursor B-ALL such as PAX5, CDKN2A, and EBF1, which indicates that these lesions represent secondary events that may be absent in the relapse-prone therapy-resistant progenitor cell. In contrast, deletions and nonsense mutations in IKZF1, which encodes the lymphoid differentiation factor IKAROS, were frequently observed and found to be consistently preserved at the time of relapse. A targeted copy number screen in the unselected cohort revealed that IKZF1 deletions are significantly associated with poor relapse-free and overall survival rates. Similar results were obtained after separate analysis of the non-high-risk and high-risk groups within this treatment protocol, i.e., non-high-risk patients with IKZF1 deletions had a ~12-fold higher relative relapse risk compared to patients without IKZF1 deletions. Consequently, IKZF1 deletion status allowed the prospective identification of 53% of the relapse-prone patients within this sub-group. *Conclusions.* In pediatric precursor B-ALL patients treated according to the dexamethasone-based DCOG-protocol ALL9 the IKZF1 deletion status serves as one of the strongest predictors of relapse at the time of diagnosis and, as such, has a high potential for future risk stratification.

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PROLONGED LEUKEMIA FREE SURVIVAL FOLLOWING BLINATUMOMAB (ANTI-CD19 BITE®) TREATMENT OF PATIENTS WITH MINIMAL RESIDUAL DISEASE (MRD) OF B PRECURSOR ALL: UPDATED RESULTS OF A PHASE II STUDY

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Background. In B-lineage acute lymphoblastic leukemia (ALL), persistence or relapse of minimal residual disease (MRD) is an independent poor prognostic factor and new treatments are needed. CD19 is the most frequently expressed B-cell differentiation antigen. It can be targeted by blinatumomab, a member of a novel class of T-cell engaging bispecific single-chain antibodies (BiTE antibodies). A Phase 2 study was conducted in collaboration with the German Multicenter Study Group on Adult Lymphoblastic Leukemia (GMALL) in order to determine the efficacy of blinatumomab in MRD-positive B-lineage ALL. *Methods.* Between May 2008 and November 2009, 21 patients with MRD persistence or relapse (MRD-level ≥ 10-4) after induction and consolidation therapy were treated with blinatumomab as a four-week continuous intravenous infusion at a dose of 15 µg/m²/d followed by a 2-week treatment-free period (1 cycle). Sixteen patients with Ph-negative (2 patients with MLL-AF4) and 5 patients with Ph-positive ALL were enrolled. MRD was assessed by quantitative reverse transcriptase-polymerase chain reaction for either rearrangements of immunoglobulin or T-cell-receptor genes, or specific genetic aberrations. *Results.* Sixteen out of 20 evaluable patients (13 out of 15 patients with Ph-negative and 3 out of 5 patients with Ph-positive ALL) became MRD-negative (MRD-level <10-4) after one cycle of blinatumomab regardless of the MRD level prior to study treatment. Nine patients were enrolled with high MRD load (> 10-2) prior to study treatment. All of these 9 patients became MRD-negative. Thirteen out of 16 patients with persistent MRD prior to study treatment and 3 out of 4 patients with MRD-relapse became MRD-negative. Nineteen patients are evaluable for duration of hematological relapse-free survival (median follow up 11 months, range from 3 to 20 months). One patient withdrew consent for follow-up. Fifteen patients remain in continuous hematological remission. Four non-transplanted patients experienced hematological relapse (3 responders and 1 non-responder). Ten responders are not transplanted after treatment with blinatumomab. Six out of these 10 non-transplanted responders are still MRD-negative without additional treatment. Six out of 7 non-transplanted responders with Ph-negative ALL are still MRD-negative without additional treatment. Eight patients (6 responders, 2 non-responders) underwent allogeneic HSCT after at least one cycle of blinatumomab (median 2 cycles) and all remain alive without hematologic relapse. Transient pyrexia (100%), chills (42.9%), headache (42.9%) were the most common clinical adverse events (AEs), which all resolved to baseline during treatment. Target-mediated Grade 3 or 4 lymphopenia (33.3%) and hypogammaglobulinemia (14.3%) were also observed, but without increased incidence of opportunistic infections. Grade 3 seizure, which was fully reversible within 1 day after drug discontinuation, was observed in 1 patient. There were no blinatumomab related deaths. *Conclusions.* Blinatumomab induces complete molecular remissions in patients with persistent and relapsed MRD independent from the extend of MRD-positivity and prolong leukemia-free survival with a favorable safety and tolerability profile. These molecular remissions might be durable without additional conventional maintenance chemotherapy.

0599

IMPACT OF ALLOGENEIC STEM CELL TRANSPLANTATION ON TYROSINE KINASE DOMAIN MUTATIONS AND MINIMAL RESIDUAL DISEASE LEVELS IN PATIENTS WITH PHILADELPHIA-CHROMOSOME-POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA

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Persistance of BCR-ABL transcripts and detection of TKD mutations during TKI based treatment of Ph+ ALL are associated with a high risk of relapse. Allogeneic stem cell transplantation (alloSCT) is potentially curative, but the ability to eradicate the different BCR-ABL mutations associated with imatinib resistance is uncertain. Moreover, the clinical implications of high mrd levels prior to SCT on outcome after SCT remains to be defined. It was the aim of this analysis to determine the impact of allogeneic SCT on MRD levels and the prevalence of BCR-ABL mutations in patients with Ph+ALL treated with imatinib in combination with chemotherapy. Bone marrow (BM) and/or peripheral blood (PB) samples were collected pre-treatment (n=26), during IM-based combination therapy prior to SCT (n=361) and serially after SCT (n=1793) from a total of 66 pts. with newly diagnosed Ph+ALL (median age: 45 yrs.) who were enrolled in prospective GMALL trials of IM followed by SCT. Additionally, we examined a total of 536 samples from 27 Ph+ALL pts. (median age: 48 yrs.) who were enrolled in the early phase II trials of IM as salvage therapy and transplanted in PR, CR2 or with progressive disease. MRD were analysed in all samples by quantitative rtPCR with nested bcr-abl PCR to confirm PCR negativity. Mutational status was analysed by denaturing high-performance liquid chromatography (D-HPLC) plus cDNA sequencing in a subset of these samples prior to and serially after transplantation, respectively. A mutation was detected at any time prior to SCT in 10 of 66 pts. (15%) with *de novo* Ph+ALL who received IM-based front-line treatment and were evaluable by both DHPLC and ASO-PCR. Of these, 4 pts. relapsed with detection of the same mutation as prior to SCT, 5 patients remain in continuous molecular CR with a median follow up of 34 months, 1 patient died in CR. 53 pts. with *de novo* Ph+ALL were evaluable prior to allogeneic SCT in CR1: of 22 pts. who achieved MRD negativity prior to SCT, 4 experienced a hematologic (n=2) or molecular relapse (n=2)[20% overall recurrence rate]. Substantially higher rates of hematologic relapse (6/18; 33%) and molecular positivity (10/18, 56%) after SCT were observed in the 18 pts. who remained MRD pos. within 6 weeks prior to SCT. Nearly all (21/23) patients with relapsed or refractory Ph+ALL who received IM as salvage treatment remained MRD positive prior to SCT. Thirteen of these 21 pts. harboured a detectable TKD mutation. Analysis of MRD and mutational status after alloSCT was possible in 8 of the 13 pts. with an initial TKD mutation. Four pts. converted to MRD-negativity, while another 4 pts. relapsed with mutated BCR-ABL. In conclusion, a strategy encompassing TKI-based frontline treatment followed by allogeneic SCT results in long-term elimination of mutant clones in the majority of patients transplanted in CR1. A minority of patients remain at risk of relapsing with previously undetectable TKD mutations, particularly after SCT beyond CR1. Some of these mutations may be responsive to second generation ABL TK inhibitors.

0600

THE NOVEL CALICHEAMICIN-CONJUGATED CD22 ANTIBODY INOTUZUMAB OZOGAMICIN (CMC-544) EFFECTIVELY KILLS PRIMARY PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA CELLS

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Background. Although conventional treatment with chemotherapy results in 80% survival in children with acute lymphoblastic leukemia (ALL), long-term side effects are frequently observed. Inotuzumab ozogamicin (CMC-544), consisting of a humanized CD22 antibody coupled to the anti-tumor antibiotic calicheamicin, specifically binds to CD22 antigens expressed on immature and mature B cells. It has recently been shown that CMC-544 is effective in patients with B-cell lymphomas and in ALL cell lines. *Aim.* To study the effectiveness of CMC-544 in primary B-cell precursor (BCP)-ALL cells *in vitro* and to analyze which parameters determine its efficacy. *Methods*. Freshly isolated or thawed BCP-ALL cells from 19 patients (15 at diagnosis, 4 at relapse) were tested for their sensitivity to CMC-544 and calicheamicin by quantitative flowcytometric measurement of viable ALL cells after 0 to 96 hours of incubation. CD22 antigen saturation, internalization of CMC-544, and renewed CD22 antigen expression were determined by flowcytometric analysis. *Results.* CMC-544 induced dose-dependent cell kill in the majority of primary BCP-ALL cells, although IC_{50} values varied substantially (median 5.4 ng/mL, range 0.1-1000 ng/mL at 48h). One patient hardly responded to CMC-544, and three patients showed a delayed response. BCP-ALL cells were at least 100 times more sensitive to CMC-544 than normal CD22- lymphocytes of the same patients and AML cells. BCP-ALL cells were highly sensitive to free calicheamicin compared to primary AML cells (median IC_{50} values at 48h: 0.21 and 1021 ng/mL, respectively). The sensitivity of BCP-ALL cells to CMC-544 was strongly related to their sensitivity to calicheamicin (R2=0.76; P<0.0001). In addition, the efficacy of CMC-544 was associated with CMC-544 internalization, and, to a lesser extent, to renewed membrane expression of CD22 antigens. No direct relation between CD22 expression levels and efficacy of CMC-544 was found, probably due to the predominant contribution of calicheamicin sensitivity to CMC-544-induced cell kill. Nevertheless, CD22low ALL patients were generally less sensitive for CMC-544 than for calicheamicin, whereas CD22high BCP-ALL cells showed comparable sensitivity for both CMC-544 and calicheamicin. Furthermore, preliminary experiments showed that in a CD22low ALL cell line the percentage of lysis after 48 hours was much lower after incubation with CMC-544 for one hour compared to continuous incubation with CMC-544, whereas this was less evident in a CD22high cell line. Additional studies in ALL cell lines showed that the cytotoxic effect of CMC-544 was mediated via induction of double-strand DNA breaks, resulting in a G2/M cell cycle arrest, and subsequent apoptosis. *Summary/Conclusions.* CMC-544 is a highly efficient and specific new drug for killing BCP-ALL cells. Since BCP-ALL cells are far more sensitive to calicheamicin than primary AML cells, they are less dependent on intracellular accumulation of calicheamicin by a repetitive loop of saturation-internalization-and renewed expression, which was required to achieve efficient Mylotarg-induced cell death in AML cells. However, high CD22 expression levels and efficient internalization of CMC-544 accelerate reaching an intracellular calicheamicin threshold level required for induction of cell death. These new data will have consequences for the design of clinical trials to test the efficacy of CMC-544 in BCP-ALL patients.

Platelets

0601

THE ROLE OF THE IAP LIVIN IN MEGAKARYOCYTES DIFFERENTIATION AND THROMBOPOIESIS

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Background. The exact mechanism of platelet production is poorly understood. A relationship between activation of the apoptotic cell machinery and the formation of pro-platelets has been established. In this study we show that Livin plays a role in this process. Livin is a member of the Inhibitor of Apoptosis Proteins (IAP) family of intracellular anti-apoptotic proteins that acts by binding and inhibiting caspases. We found that Livin is unique among the IAP members as upon strong apoptotic stimuli it is specifically cleaved by caspases to produce a truncated protein (tLivin) with a paradoxical pro-apoptotic activity. *Methods.* In this work we studied Livin expression in normal bone marrow (BM) and in BM from patients with hematological diseases using immunohistochemistry (IH) staining. An *in vitro* model was established to evaluate the potential role of Livin in thrombopoiesis. The human BCR-ABL positive cell line, LAMA-84, was induced by a phorbol ester (PMA) to differentiate to MK. The effect of PMA on megakaryocytic differentiation of LAMA-84 cells was investigated both by morphological changes and by the expression of CD41 and CD71. Increased cell size, ploidy and DNA synthesis, all markers of MK differentiation, were detected by flow cytometry (FACS). *Results.* Livin protein was clearly detected in MK in normal mature BM (by IH staining) and is expressed in platelets. Livin expression was also demonstrated in MK of patients with various hematological diseases such as ITP, MDS, Hodgkin's disease, ET and PV. Differentiation was characterized by up regulation of the MK marker CD41 from 6% to >60% and down regulation of the erythroid cell marker CD71 from 97-70%. Upon differentiation induced by PMA, LAMA-84 cells formed pro-platelets and produced functional platelets capable of aggregation. This differentiation of LAMA-84 cells into the MK was accompanied by Livin protein expression. In contrast to Livin, the anti-apoptotic proteins Bcl-2, XIAP and Survivin levels decreased upon MK differentiation. Moreover, when we derived single clones from the LAMA-84 cell line and induced differentiation of these clones with PMA, only clones that expressed Livin upon differentiation produced functional platelets that aggregated in response to stimulators. At the terminal stage of differentiation and platelets production, we observed a drastic decrease in the anti-apoptotic full length form of Livin and accumulation of the pro-apoptotic tLivin concomitant with increased caspase 3 activity and apoptosis. *Summary/Conclusions.* We established an *in vitro* model to investigate the role of Livin in thrombopoiesis. LAMA-84 cells differentiate into megakaryocytic lineage and produce functional platelet like particles. The IAP Livin is upregulated upon MK differentiation and is then cleaved to pro-apotic tLivin at the end of this process. We suggest that Livin plays a role in thrombopoiesis by regulating the apoptotic cell machinery in MK.

0602

AN ENU-DERIVED MOUSE MODEL OF MYH9-RELATED DISEASE IDENTIFIED BY MASSIVELY MULTIPLE PARALLEL SEQUENCING

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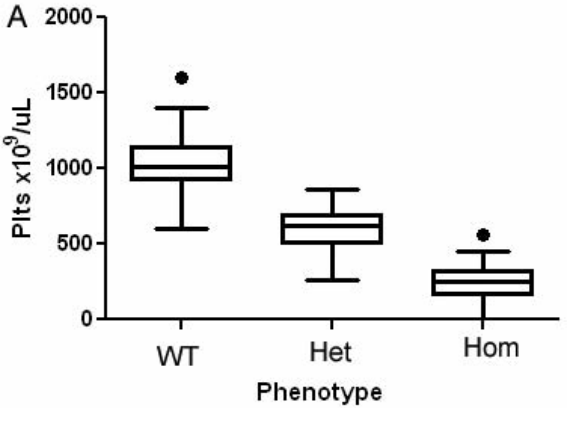
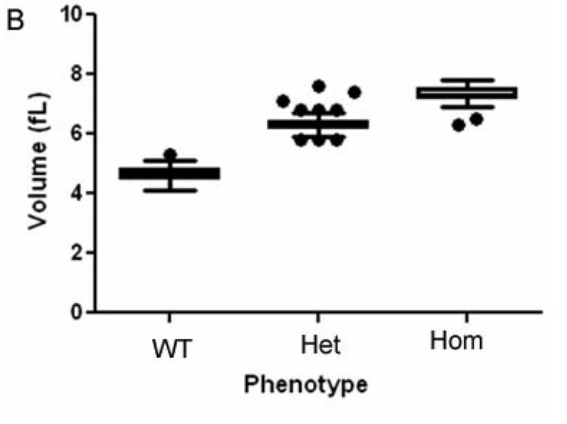
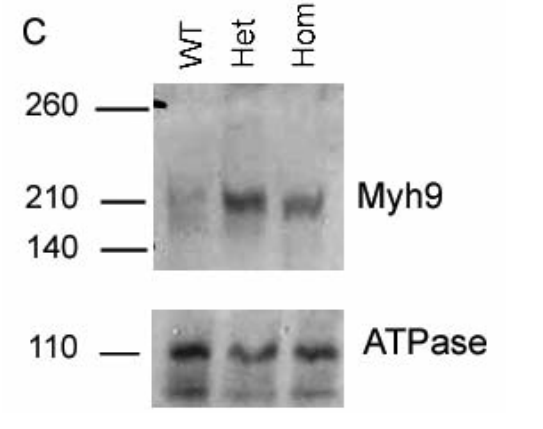
Background and *Aims.* To produce models of human hematopoiesis, we induced random point mutations using N-ethyl-N-nitrosourea (ENU) mutagenesis in 129S1/SVImJ (129) male mice, which were bred to C57BL/6J (B6) females. Offspring were monitored for deviations in peripheral blood composition and backcrossed to B6 mice to determine heritability and positionally clone the mutated gene. The mutant strain 7238 presented with macrothrombocytopenia, as the peripheral blood of unaffected mice averaged a platelet concentration of 1031±169×10^9 plt/μL and a mean platelet volume (MPV) of 4.7±0.2fL; heterozygous mice, 599±126×10^9 plt/μL and 6.3±0.3fL; and homozygous mice, 245±109×10^9 plt/μL and MPV of 7.3±0.2fL (Figure 1A and B).







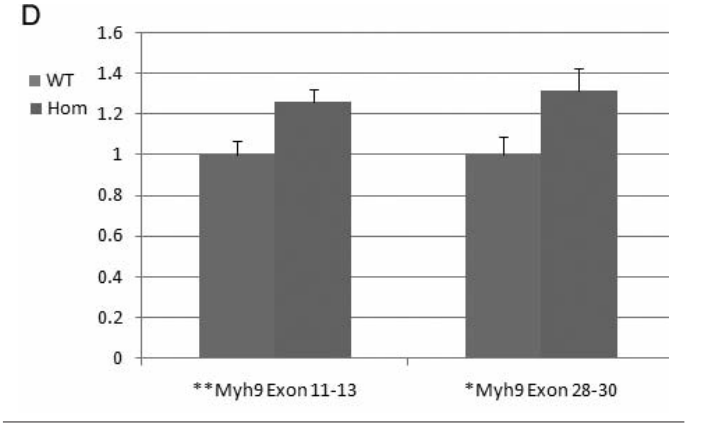


Figure 1. Phenotyping data for the Myh9Q1443L mouse line.

Methods. We used array-based sequence selection followed by massively multiple parallel sequencing to identify potential mutations within our 4.25Mb region of interest, located on chromosome 15. We designed a custom NimbleGen array for covering the 4.25Mb interval using overlapping 25-mers unique within the interval. Genomic DNA was sheared to ~200bp and hybridized to the array. Following washing, the DNA was denatured and sheared to 25-50bp, and linkers were ligated. These sequences were bound to the Illumina lawn, amplified in place, and concurrently sequenced *in situ* using temporarily terminated, fluorescently labeled dNTPs, and assembled using the B6 mouse sequence as a reference. Illumina sequencing identified 19 potential mutations located within the exon and border regions of 7238. Of these, the only non-synonymous coding is in exon 30 of Myh9. Sanger sequencing of both the 129 and B6 parental lines has shown that this site is not a novel single nucleotide polymorphism (SNP), and that Q1443L is an ENU-induced mutation. Genotyping of offspring has shown that this mutation is indicative of the observed macrothrombocytopenia phenotype. Further, we are sequencing the parental strains for the remaining 18 potential mutations; we have currently demonstrated that eleven of these sites are novel B6/129 SNPs. Therefore, the 7238 strain is relabeled Myh9Q1443L, and is a model of MYH9-related disease (MYH9-RD). *Results.* Human MYH9-RD is characterized by macrothrombocytopenia and various combinations of neutrophil inclusions, increased bleeding and bruising, cataracts, nephritis, and sensorineural deafness. The tail bleed assay demonstrated while both the wild type and Myh9Q1443L/+ mice had similar clotting times (75±36s and 98±79s, respectively, P=0.17), the Myh9Q1443L/ Q1443L mice take significantly longer (319±276s, P<0.005). We have also shown that the expression level of Myh9 protein and transcript is consistent between genotypes (Figure 1C, D). Experiments are underway - including neutrophil studies, urine analysis and auditory brainstem response studies - to determine which clinical presentations of MYH9-RD are characteristic of the Myh9Q1443L mouse model. Data from these studies will be presented. *Conclusions.* Upon completion of these experiments, we will have produced and characterized the first mouse model capable of modeling multiple aspects of MYH9-RD. This model will therefore be a valuable tool to better understand the function of MYH9 in various tissues, and potentially lead to treatments aimed at lessening the clinical symptoms of this disorder.

0603

EFFICACY OF RITUXIMAB IN COMBINATION WITH DEXAMETHASONE VS DEXAMETHASONE IN NEWLY DIAGNOSED PATIENTS WITH PRIMARY IMMUNE THROMBOCYTOPENIA - AN INTERIM ANALYSIS OF A PROSPECTIVE RANDOMISED STUDY

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Background. Primary immune thrombocytopenia (ITP) is characterised by an immune-mediated destruction of platelets and impaired platelet production. Glucocorticoids, including dexamethasone, are the recommended first-line therapy for newly diagnosed ITP patients. Several studies have shown that rituximab induces an increase in platelet count in up to 60% of patients with refractory ITP. *Aims.* This study provides the interim data from an on-going national multicenter study comparing rituximab in combination with dexamethasone vs dexamethasone as monotherapy in treatment of newly diagnosed ITP patients. *Methods.* Patients are enrolled at 10 Danish haematology departments according to predefined criteria. All patients have given written informed consent prior to enrolment. Patients are ≥ 18 years of age with confirmed ITP and platelet counts ≤ 25×10^9/L or ≤ 50×10^9/L in the presence of bleeding symptoms. Eligible patients are randomised 1:1 by use of pre-coded envelopes to receive either dexamethasone (40 mg daily for 4 consecutive days repeated every 1-4 weeks for a total of up to 6 treatment cycles) as monotherapy or in combination with rituximab(375 mg/m^2 repeated once weekly for a total of 4 weeks. Pre-medication includes paracetamol and antihistamine to reduce infusion-related discomfort). Primary endpoint is sustained complete response (CR, platelets ≥100×10^9/L) or partial response (PR, platelets ≥ 50×10^9/L) at 6 months follow-up. Secondary end-points include relapse-free time periods and rates of splenectomy. Adverse effects are reported and graded by the individual clinician. Response rates, frequency of splenectomy and adverse effects were analysed by the Fisher exact test. Relapse-free survival was calculated using the Kaplan-Meier method.

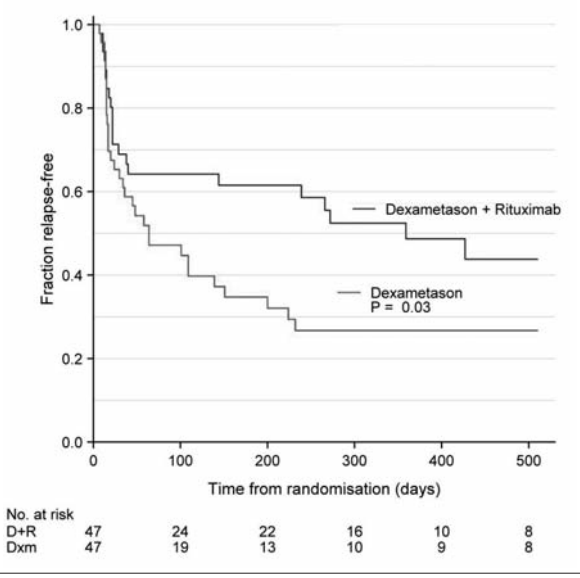

Figure 1. Kaplan-Meier plot of relapse-free survival.

Results. 116 patients were assessed for eligibility, 13 patients were excluded according to predefined criteria and 9 patients were excluded from these interim analyses due to lack of data. 94 patients were included in the efficacy and toxicity analysis (median age 51 years, range 18-84, 59% women). Sustained response (CR or PR) at 6-months follow up was achieved in 55% of patients in the rituximab group and 34% of patients in the dexamethasone monotherapy group (P=0.1). Fraction of relapse-free patients during the course of the study (up to 500 days) was significantly higher in the rituximab+dexamethasone group than in the dexamethasone group (P=0.03, Figure 1) There was no significant difference in splenectomy-rates between the two groups (2 patients in the dexamethasone group, 5 patients in the rituximab+dexamethasone group, P=0.3). Adverse effects were mild to moderate, grade 1-2 adverse effects were reported in 85% of patients in the dexamethasone group and 74% in the rituximab+dexamethasone group (P=0.32). Grade 3 adverse effects were reported in 4 patients in the dexamethasone group and 9 patients in the rituximab+dexamethasone group, 3 of these patients had 2 events (P=0.32). *Conclusion.* Initial treatment of newly diagnosed ITP patients with rituximab in combination with dexamethasone induced a sustained response in 55%, and gave rise to significantly longer relapse-free survival than in patients treated with dexamethasone alone.

0604

RESULTS OF BONE MARROW EXAMINATIONS IN PATIENTS WITH CHRONIC IDIOPATHIC (IMMUNE) THROMBOCYTOPENIC PURPURA TREATED WITH ELTROMBOPAG FOR MORE THAN ONE YEAR

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Background. Increased reticulin fibers can be found in bone marrow of patients with neoplasms and autoimmune diseases, and can lead to a condition similar to primary idiopathic myelofibrosis ([MF]; Frisch Haematol [Budap] 1982; Aharon Lupus 1997; Mufti J Supp Onc 2007). In healthy individuals, grade 1 reticulin has been reported in 27%-70% of bone marrow biopsies, while grade 2 reticulin has been reported in 4%-20% (Hultdin Med Onc 2007; Beckman Arch Path Int Med 1990; Bauermeister Am J Clin Path 1971). Bone marrow biopsies are not routinely performed in patients with chronic idiopathic (immune) thrombocytopenic purpura (ITP). Based on limited data published prior to the introduction of thrombopoietin receptor agonists, grade 1-2 bone marrow reticulin was reported in up to 67% of patients with ITP (Mufti 2007). Theoretically, stimulation of megakaryocytes with thrombopoietin receptor agonists may increase the risk of bone marrow fibrosis. Reticulin deposition, peripheral erythroblasts, tear drop cells, and blasts were reported in patients with chronic ITP treated with romiplostim (Kuter Blood 2009). Eltrombopag is the first oral, nonpeptide thrombopoietin receptor agonist licensed for the treatment of chronic ITP. EXTEND is an ongoing, long-term open-label ITP study in which patients who completed previous eltrombopag trials receive eltrombopag at a starting dose of 50 mg. *Aims.* To determine whether eltrombopag treatment is associated with a clinically significant increase in bone marrow reticulin and collagen *Methods.* In EXTEND, a bone marrow biopsy is requested annually while on treatment. Complete blood counts and white blood cell (WBC) differentials are performed at each visit. If immature or dysplastic cells are identified, a peripheral blood smear is performed. If abnormal cells are confirmed, a bone marrow biopsy is indicated; a bone marrow biopsy can be performed any time at the investigator's discretion. Reticulin is quantified using the modified MF scale (Thiele Haematologica 2005). *Results.* As of February 1, 2010, 135 patients treated for a median of 12 months at the time of the procedure (range: 1-32 months) had bone marrow biopsies evaluated for reticulin/collagen. The summary of the reticulin and collagen findings from on-treatment biopsies is presented in the Table. None of the 135 patients experienced a reticulin grade of MF-3; 3 had collagen. Of the 11 patients with a reticulin grade of MF-2, none showed clinical signs or symptoms that would indicate bone marrow dysfunction (ie, abnormal WBC differential or peripheral blood smear). Eleven of the 135 patients had a second on-treatment biopsy after ≥24 months. Compared to the first on-treatment biopsy, 8/11 patients had no change in reticulin grade, 1/11 experienced an increase of MF-1 to MF-2; and 2/11 experienced a decrease in reticulin grade (MF-2 to MF-0 and MF-1 to MF-0). No biopsy was prompted by clinical symptoms or a blood smear suggestive of

MF. *Conclusions.* In this, by far the largest series, patients treated for ≥1 year with eltrombopag had no evidence of clinically relevant increases in bone marrow fibers. Pretreatment bone marrow biopsies need to be considered in future studies.

Table.

Parameter	Patients With On-Treatment Bone Marrow Biopsies* N=135
Evaluable samples examined for reticulin/collagen, N[a]	146
MF grade 0 (or "no", "no significant increase", "no evidence of fibrosis")	87
MF grade 1 (or focal mild)	48
Collagen reported	1[b]
MF grade 2	11[b, c]
No collagen reported	9
Collagen reported	2[b]
MF grade 3	0
*Median duration of eltrombopag treatment, months: 12(1–32) for MF grades 0 and 1; 15(12–24) for MF grade 2.	

MF, myelofibrosis.

a. 11 patients had 2 samples (after 12 and 24 months of treatment).
b. No symptoms potentially related to bone marrow dysfunction.
c. 2/11 patients had reticulin in pretreatment bone marrow biopsies, considered to have increased on study. Pretreatment biopsies were unavailable for the vast majority of patients.

0605

THE EFFECT OF DESMOPRESSIN (DDAVP) ON PLATELET FUNCTION: SELECTIVE AND MARKED ENHANCEMENT OF PLATELET PROCOAGULANT ACTIVITY

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Background. Desmopressin (DDAVP) is clinically efficacious in patients with disorders of the platelet function. This effect might be mediated by the release of von Willebrand factor (VWF) from endothelial cells. However, the documented efficacy of DDAVP in patients with Bernard-Soulier suggests that other mechanisms may be involved. *Aim.* To evaluate the effect of *in vivo* DDAVP administration on platelet function, in particular on aggregatory and procoagulant properties. *Methods.* We performed platelet aggregation (PA) and flow cytometry in 31 patients with disorders of the platelet function who received a test dose of DDAVP (0.3 mcg/kg body weight). Following platelet characteristics were studied (at baseline and 2-4 hours after DDAVP): 1) PA induced by ADP, collagen, arachidonic acid and ristocetin. 2) Surface expression of glycoprotein (GP) Ib and GPIIb-IIIa. 3) Content of delta-granules and their secretion induced by thrombin (T). 4) Secretion of alpha-granules and GPIIb-IIIa activation by graded concentrations of ADP, convulxin (CVX, a specific agonist of the collagen receptor GPVI) or T. 5) Generation of procoagulant COAT platelets induced by CVX and T. 6) Platelet-dependent thrombin generation (TG) assessed by an in house modification of the Hemker's method. *Results.* Agonist-induced PA was unchanged after DDAVP-infusion. Surface GPIb and GPIIb-IIIa did not significantly change. Delta-granules content and their T-induced secretion remained unchanged. Secretion of alpha-granules and GPIIb-IIIa activation by ADP significantly decreased by 40% and 18% respectively; both end-points induced by CVX decreased by 15% and 6%; both end-points induced by T decreased by 12% and 7%. Of note: 5) Percentage of COAT platelets significantly increased by 27%, and 6) Platelet-dependent TG significantly increased by 30%. *Conclusions.* We show that *in vivo* administration of DDAVP selectively and markedly enhances the ability to form COAT platelets and increases platelet-dependent TG, while it does not improve platelet aggregation. This is the first report showing that the beneficial haemostatic effect of DDAVP is not limited to an increase in large VWF multimers. An enhancement of platelet procoagulant activity appears to be an additional and -at least in platelet disorders- possibly more important mechanism of DDAVP's action.

POSTER SESSION II

Acute lymphoblastic leukemia - Clinical

0606

MICRORNA EXPRESSION IN PEDIATRIC PRECURSOR-B ACUTE LYMPHOBLASTIC LEUKEMIA: BIOLOGY, CLASSIFICATION AND PROGNOSIS

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Background. B-lineage acute lymphoblastic leukemia (ALL) consists of ~80% of childhood ALL cases and is characterized by different genetic subgroups based on specific recurrent genetic abnormalities, bearing prognostic and therapeutic implications. MicroRNAs (miRNAs) are a class of small endogenously expressed repressor RNAs that play key roles in many cellular pathways. Recently, miRNA expression patterns have been linked to cancer subtypes as well as to disease outcome. *Aims.* In this study, we aimed to define whether different genetic subgroups in pediatric precursor-B ALL are characterized by a distinct miRNA expression signature. Furthermore, we assessed whether miRNA profiling might provide useful information for better classification and risk stratification and additionally, might contribute to our understanding of the underlying disease biology. *Methods.* A total of 693 miRNAs were profiled using automated high-throughput quantitative stem-loop RT-PCR in a cohort of 48 average risk (AR1) pediatric precursor B-cell ALL patients treated according to EORTC protocols 58881 and 58951. The cohort included cases with normal karyotype (N=6), hyperdiploid karyotype (N=12), TEL-AML1 (N=20), E2A-PBX1 (N=3) and complex rearrangements (N=7). The miRNA profiles of mononuclear cells from 13 normal bone marrow samples served as control. Moreover, a microRNA expression map of four FACS-sorted B-cell subsets was generated. The ethical committee approved the study and informed consent was obtained from the patients and/or their parents. *Results.* MiRNA profiling of the sorted B-cell subsets revealed that 97 out of 693 miRNAs were significantly expressed to more or less extent in one or more B-cell subsets. In more detail, we found that 16 miRNAs are exclusively expressed in one stage of early B-cell development, whereas the remaining showed periodic activity during B-cell maturation. Further, Significance Analysis of Microarray (SAM) revealed a 36 miRNA signature (FDR = 5%), able to distinguish between the different genetic subgroups (TEL-AML1, E2A-PBX1 and Hyperdiploidy) after unsupervised hierarchical clustering. Using Prediction Analysis for Microarrays (PAM, using cross-validation), we identified a specific and sensitive minimal classifier of 12 miRNAs for the above mentioned pre-B ALL subtypes. Adding the 'complex' sample profiles revealed that many of them clustered together with the E2A-PBX1 subgroup, suggesting a common molecular background. Target prediction of differentially expressed miRNAs revealed an overrepresentation of genes involved in cell cycle, MAPK signaling, WNT signaling and TGF-β signaling. Finally, preliminary results suggest that miRNA expression differences observed at diagnosis could give useful information on the probability of relapse. The outcome signatures are currently being validated in a larger, independent cohort. *Conclusions.* This study shows that cytogenetic subgroups in pre-B ALL are characterized by a specific miRNA expression signature. The generation of a minimal classifier within the AR1 group will allow addressing the 'normal' and 'complex' samples into a better defined subgroup. Correlation of our findings to the expression of deregulated miRNAs in normal B-cell subsets will point us to the miRNAs with true oncogenic potential and allows delineating a subset for further functional studies. After stringent validation, the preliminary outcome signature might guide us to a better understanding and handling of the possibility to relapse.

0607

CLINICO-BIOLOGIC CHARACTERIZATION OF 5203 ACUTE LYMPHOBLASTIC LEUKEMIA PATIENTS ENROLLED IN THE ITALIAN AIEOP AND GIMEMA PROTOCOLS, STRATIFIED IN AGE-COHORTS FROM SMALL CHILDREN TO YOUNG ELDERLY

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Background. Acute lymphoblatic leukemia (ALL) shows marked differences in clinical outcome between children and adults. Several factors account for this different scenario, including the variable incidence of molecular aberrations. It is likely that other factors differ between the two cohorts. Overall, there is limited information on the distribution of the clinico-biologic variables in the various age-cohorts, ranging from small children to young elderly. *Aims.* To define the clinico-biologic profile of newly-diagnosed ALL patients, stratified in 9 age-cohorts: 1-5, 5-10, 10-14, 14-18, 18-25, 25-30, 30-40, 40-50, 50-60 years. *Methods.* Overall, 5203 patients were evaluated: 3754 children enrolled in the AIEOP protocols and 1449 adults recruited in the GIMEMA protocols. Clinical features included WBC (>50×10^9/L) and Plt (<100×10^9/L) counts, Hb levels (<10 g/dL), liver, spleen, mediastinum enlargement and CNS involvement. Biological features comprised immunophenotypic and molecular analyses. Differences among groups were evaluated using non-parametric tests (Chi-squared and Fisher-Exact test). *Results.* There were 2890 males and 2313 females (M/F=1.25).The highest incidence was recorded in the age-cohorts 1-5 and 5-10 (36.6% and 21.4%, respectively), with a progressive decrease from 10 to 30 years and an increase from 30 years onwards. Gender distribution highlighted a lower incidence of females: this phenomenon was remarkable between 14-50 years and disappeared in the 50-60 age-cohort (P<.0001). Hyperleukocytosis was less frequent in the age-cohorts 1-5 and 5-10 (P<.0001); spleen, liver and mediastinum enlargement were present in 28.8%, 19.5% and 7% of cases, respectively: there was a constant decrease of organ involvement in older patients (P=0.0276, P<.0001, P<.0001). CNS involvement was recorded in 2.1% of cases, most frequently between 1-14 years. A T-lineage affiliation was rarer among the 1-5 and 5-10 age-cohorts (5.1%, and 15.7%, respectively), increased in patients between 10 and 40 years (average=25%), and decreased in the elderly (40-50=11.9%, 50-60=12.2%). There was a significant association between gender and immunophenotype, T-ALL being more frequent in males (P<.0001) up to the 4th decade. A B-lineage derivation was found in 85.8% of patients: an increased incidence of pro-B ALL, sustained by ALL1/AF4 positivity, was recorded in the age-cohorts from 10 to 50 years. Molecular screening highlighted a progressive increase in BCR/ABL$^+$ cases (P<.0001), detected in 52.7% of B-lineage cases in the 6th decade, and in ALL1/AF4+ cases with age (P<.0001) and a constant decrease in ETV6/RUNX1+ cases (P<.0001), whereas E2A/PBX1+ cases showed a consistent distribution among age-cohorts.

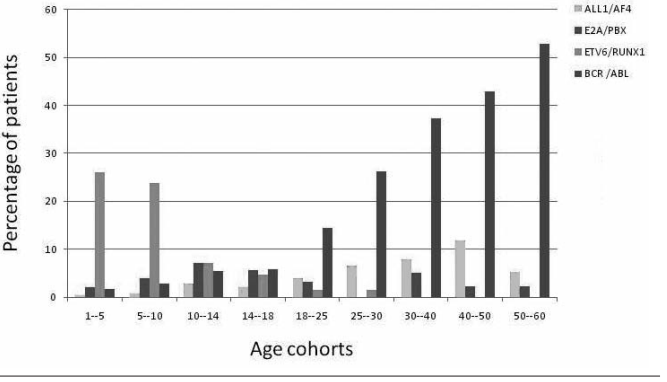

Figure 1. Molecular aberrations incidence among age-cohorts

Conclusions. Stratification in 9 age-cohorts confirms a high incidence of ALL in small children and shows a lower occurrence of ALL in females, particularly significant between 14-50 years, suggestive of a protective effect of fertility. Organ involvement declines with age, pos-

sibly reflecting a progressive, physiological atrophy, that may result in a lesser efficient immunologic control of the disease, ultimately translating in a worse outcome Finally, we conclusively quantify the decrease of ETV6/RUNX1 and progressive increase of ALL1/AF4 and BCR/ABL with age. This results in an increase with age of unfavorable prognostic markers that may be managed by targeted therapies, bearing in mind that in the 50-60 age-cohort BCR/ABL positivity accounts for over 50% of B-lineage cases.

0608

IDENTIFICATION OF A FAVORABLE PROGNOSIS GROUP OF ADULT B-LINEAGE ACUTE LYMPHOBLASTIC LEUKEMIA WITHOUT MOLECULAR ABERRATIONS BY COMBINING GENE EXPRESSION AND SINGLE NUCLEOTIDE POLYMORPHISM ARRAYS

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Background. In B-lineage acute lymphoblastic leukemia (B-ALL) several rearrangements (BCR/ABL, MLL/AF4, E2A/PBX1) have been identified and have a prognostic impact. However, roughly 50% of cases lack major genetic aberrations visible by conventional karyotyping; for these patients the therapeutic approach is largely empiric, resulting in over or undertreatment. In B-ALL, the introduction of new advanced technologies, i.e. Single Nucleotide Polymorphism (SNP) analysis and Gene Expression Profiling (GEP), have greatly the improved discovery of novel recurrent lesions and the identification of novel prognostic subsets, especially in the pediatric setting. *Aims.* To highlight recurrent genomic aberrations in adult B-ALL, we performed SNP array analysis on a cohort of newly diagnosed cases lacking known molecular rearrangements and we integrated these data with GEP results and clinico-biologic features. *Methods.* For SNP analysis, 38 B-ALL samples without major molecular rearrangements were genotyped with the GeneChip® Human Mapping 250K NspI array (Affymetrix). Analyses were performed using the Partek Genomic Suite 6.4 and dChip softwares. Copy number aberrations were scored using the Hidden Markov Model and the segmentation approach. For GEP experiments, we evaluated the same samples and an additional cohort of 33 patients using HGU133 Plus 2.0 arrays (Affymetrix). Statistical analysis, performed using the dChip software, was based on unsupervised and supervised approaches; DAVID software was used for functional annotation analysis. Overall survival (OS), disease-free survival (DFS) and event-free survival (EFS) were estimated using the Kaplan-Meier product limit method and compared in univariate analysis using the Log-Rank test. All analyses were run in SAS. *Results.* Sixty-eight percent of cases displayed alterations larger than 1.5 Mb, the most frequent being deletions of 9p (32%), 20q (16%), 12p (16%), 7p (13%) and gains of chromosome 21 (21%). In a large proportion of patients (88%), small alterations (<1.5 Mb) were documented, the most common involving IKZF1 (58%) and CDKN2A/2B (39%), whose deletions produced a gene dosage effect. Deletion of the IFNA cluster genes, often associated with loss of MLLT3, was also frequent (32%). GEP analysis, namely unsupervised clustering, revealed the presence of several subsets (Cluster I-V). Remarkably, when these data were integrated with SNP results, an association between Clusters and specific large alterations was observed (P=0.05). Correlation with clinical variables and outcome was highly revealing: Cluster II, lacking by SNP analysis large alterations, was characterized by a lower white blood cell (WBC) count at presentation (19.1×10^9/L *vs.* 52.2×10^9/L, mean of all other Clusters), a better EFS (P=0.0015) and DFS (P=0.0003). By Linear Discriminant Analysis, additional cases falling in the above mentioned Clusters were identified and statistical analysis of clinical data confirmed that patients included in Cluster II experience a better outcome. Moreover, functional annotation clustering performed on ANOVA result highlighted an enrichment in mitochondrial genes (BH: 7.8E-1) in Cluster II specific genes. *Conclusions.* In summary, most adult B-ALL cases without major genetic abnormalities harbor copy number alterations when examined by refined techniques. Moreover, the integration of GEP and SNP data is highly informative, allowing to identify a subset of patients with a very favorable outcome.

0609

EFFECTS OF GLUCOCORTICOID RECEPTOR GENE N363S POLYMORPHISM ON THE STEROID - INDUCED TOXICITIES DURING THE PEDIATRIC ALL THERAPY

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Introduction. Glucocorticoidreceptor (GR) gene polymorphisms are reported to be associated with increased glucocorticoid (GC) sensitivity. For that reason GR gene polymorphisms may modify the severity of the adverse effects of glucocorticoids during the chemotherapy of children with acute lymphoid leukemia (ALL). *Aim.* In our study we aimed to investigate whether any relation could be marked between the N363S GR gene polymorphism and acute glucocorticoid-induced side effects in children with ALL during their chemotherapy. *Patients and Methods.* In this retrospective study 346 pediatric patients with ALL were involved. DNA was extracted from peripherial blood taken in heamatologic remission. N363S polymorphism was determined by allele-specific PCR. Most common glucocorticoid toxicities were investigated such as hepatotoxicity, glucose metabolism abnormalities, encephalopathy, elevated blood pressure. *Results.* 32 of the 346 pediatric patients found to be heterozygous carriers (9.2%). N363S polymorphism was found to be related to hepatotoxicity and glucose metabolism abnormalities. Patient with the 363S variant had a significant higher risk for hepatotoxicity than the non carriers (31.2% *vs.* 11.1%, P=0.004). Also glucose metabolism abnormalities occurred more often among carriers than non carriers (18.7% *vs.* 3.7%, P=0.001). In our study there was no significant association between the patients with and without the polymorphism regarding the occurrence of encephalopathy (8.6% *vs.* 6.25%, P=1.0) or hypertension (28.1% *vs.* 18.1% P=0.171). The numbers of patients with at least one toxicity (65.6% *vs.* 34.1%, P=0.001) were significantly higher in the groups of carriers. Our results were similar regarding patients having at least two (21.9% *vs.* 6.7% P=0.009), or at least three (9.4% *vs.*1.3% P=0.02) glucocorticoid-induced toxicities. Their numbers were significant elevated among the 363S-carriers. *Conclusion.* Patients with N363S polymorphism may prone to have more and also more sever glucocorticoid-induced side effects during the chemotherapy. These results suggest the prospective opportunity of an individual dosing of chemotherapeutic drugs.

0610

ONE YEAR REINDUCTIONS WITH VINCRISTINE, ASPARAGINASE AND PREDNISONE AS POST-REMISSION THERAPY IMPROVES OUTCOME IN CHILDREN WITH INTERMEDIATE RISK (IR) ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

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Background. The ALL89 PETHEMA trial proved that delayed consolidation had a favorable influence on the outcome of children with IR-ALL.The subsequent ALL96 trial incorporated reinductions with vincristine, asparaginase and prednisone (VAP) during the first year of maintenance therapy to consolidations of the previous protocol. *Aims.* We compared both cohorts of children to evaluate the benefit of such approach in children with newly diagnosed ALL of low (LR) and intermediate risk (IR) in first complete remission (CR1). *Methods.* Children aged 1 to 18 years were classified as LR if they were 1 to9 years of age and the WBC at diagnosis was $<50\times10^9$/L and as IR if their age was >9 to 18 years or the WBC was $>50\times10^9$/L. High-risk patients [i.e. children with ALL carrying the t(9;22) or t(4;11) translocations or with WBC > 200×10^9/L] were excluded from analysis. The ALL89 trial assigned 213 with LR (N=111) or IR (N=102) ALL in CR1 to receive early or early and late consolidation cycles followed by 2 years of maintenance treatment with oral mercaptopurine and weekly intramuscular methotrexate. Children (86 IR and 233 LR) achieving CR in the ALL96 trial received similar early and delayed consolidations plus monthly vincristine (2 mg IV), asparaginase (20000 UI/sq meter IV) and prednisone (60 mg/sq meter PO x 7 days) reinductions (VAP) during the first year of maintenance treatment. The primary end point of the historical comparison was disease-

free survival (DFS). *Results.* Median follow-up of children in CR1 was 6 years. Patients in both trials were comparable in demographic and ALL characteristics. 109 children (75 LR and 34 IR) in the ALL89 trial were assigned to receive early consolidation only (Cohort 1 - C1) and 104 (36 LR and 68 IR) to receive early and delayed consolidation (Cohort 2 -C2). 281 children (79 LR and 202 IR) in the ALL96 trial received a median of 7 (range 3-7) reinductions with VAP after both consolidations (Cohort 3 - C3). 46 LR patients relapsed (24 in C1, 9 in C2 and 13 in C3). The 5-year DFS rates were 67% (95%CI 58-76), 74% (95%CI 62-86) and 84% (95%CI 77-93) for C1, C2 and C3 respetively. The hazard ratios for patients in the C2 and C3 groups were 0.77 (95%CI 0.36-1.65, P=NS) and 0.45 (95%CI 0.22-0.93, P=0.031). 80 IR patients relapsed (18 in C1, 27 in C2 and 25 in C3). The 5-year DFS rates were 46% (95%CI 31-61), 57% (95%CI 46-68) and 80% (95%CI 75-86) The hazard ratios for patients in the C2 and C3 groups were 0.77 (95%CI 0.42-1.39, P=NS) and 0.29 (95%CI 0.16-0.52, P<0.001) respectively. Two children (1 in the C2 and 1 in the C3 group) died in CR1 due to consolidation or reinduction related toxicity. *Conclusions.* VAP reinductions significantly reduced the relapse rate and improved the DFS of children with LR and IR ALL in CR1. The major benefit was obtained in IR ALL.

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0611

EFFICACY AND CLINICAL OUTCOME OF PHILADELPHIA (PH) POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) PATIENTS TREATED WITH SECOND GENERATION TYROSINE KINASE INHIBITORS (TKIS): THE BOLOGNA EXPERIENCE

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Background. Approximately 30% of adult ALL patients are characterized by the presence of the Philadelphia (Ph) chromosome, which derives from a reciprocal translocation t(9;22)(q34;q11) and results in a chimeric BCR-ABL oncogene. The prognosis for this subset of patients treated with standard therapies, including multi-agent chemotherapy, imatinib, and allogeneic stem cell transplantation, is still dismal, due to a high risk of relapse. Dasatinib and nilotinib are second generation TKIs developed to overcome resistance to imatinib in relapsed Ph^+ leukemias. Here, we sought to determine the efficacy of these agents in patients with relapsed Ph^+ ALL. *Methods.* We retrospectively evaluated the single-center experience of dasatinib, nilotinib, and experimental third-generation TKIs administered as second or subsequent lines of therapy. We evaluated the efficacy of these agents in 25 adult patients with relapsed Ph^+ ALL. All patients were previously treated with imatinib. *Results.* The median age at time of diagnosis was 50 years (range, 18-74 years). Seventeen patients were male and 8 were female. Ten patients presented with a BCR-ABL P190 fusion protein and corresponding fusion transcript, and the remaining patients exhibited BCR-ABL P210. Nineteen patients received dasatinib, 2 patients nilotinib, and the remaining 4 patients were treated with third-generation TKIs. Fourteen patients (56%) were in first relapse, and 7 (28%), 3 (12%) and 1 (4%) were in second, third, and fourth relapse, respectively. A mutational analysis was performed twice in all the patients: before administering TKIs (9 patients with wild type BCR-ABL, 16 with mutated BCR-ABL, including T315I) and at the time of subsequent relapse. Gene expression profiling, SNPArray (6.0 Affymetrix chip), and Ikaros deletions were also analyzed. Thirteen out of 25 patients (52%) achieved a hematologic response (HR) (11 patients treated with dasatinib, 1 patient with nilotinib, and 1 patient with a third-generation experimental TKI). Ten patients also achieved a cytogenetic response (CyR) and 6 patients a molecular response (MolR). With a median follow up of 10.8 months (range, 2-29 months), median duration of HR, CyR, and MolR was 117 days (range, 14-385 days); progression free survival was 162 days with dasatinib and 91 days with nilotinib. Overall survival was 25.8 months. Interestingly, in 6 out of 9 patients with wild-type BCR-ABL treated with dasatinib, the mutational analysis showed the emergence of T315I or F317I mutation at the time of relapse. *Conclusions.* Second- and third-generation TKIs represent a valid approach in treating patients with relapsed Ph^+ adult ALL. The subsequent relapse in Ph^+ ALL patients is often associated with the emergence of mutations conferring resistance to TKIs. *Acknowledgments*. European LeukemiaNet, AIL, AIRC, Fondazione Del Monte di Bologna e Ravenna, FIRB 2006, PRIN 2008, Ateneo RFO grants, Project of integreted program (PIO), Programma di Ricerca Regione - Università 2007-2009.

0612

ASPARAGINASE LEVELS, ANTIBODIES AND SILENT INACTIVATION IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction. Asparaginase is a cornerstone in the treatment for childhood acute lymphoblastic leukemia (ALL). Depletion of circulating asparagine by asparaginase results in selective apoptosis of leukemic cells who require large amounts of this circulating amino acid for their cell growth. Asparaginase of bacterial origin, like E. coli asparaginase (Paronal®), induces anti-asparaginase neutralising antibodies in a large proportion of the population. This results in allergic reactions or silent inactivation, characterized by circulating antibodies and rapid clearance of the enzyme and probably impairment of the outcome by inappropriate efficacy of this potent anti-leukemic drug. *Aim of the study.* Monitoring of asparaginase levels in children with ALL, detection of antibodies and identification of silent inactivators, in order to elucidate their impact on the outcome of these patients. *Materials and Methods.* Between May 2004 and August 2008, we collected serum samples of 104 patients with *de novo* ALL who received asparaginase according to the EORTC 58951 protocol. Asparaginase enzymatic activity was measured by photometry at 450nm after reaction with Nessler's reagent. Quantitative detection of human anti-E. coli asparaginase antibodies was performed by using a direct ELISA format. Asparaginase activity >100 U/L was considered to be sufficient for complete depletion of asparaginase in the serum. *Results.* 51 (49%) of the 104 patients developed an allergic reaction to Paronal®, 26 of them were boys, 25 girls. 4 were treated according to the very low risk group, 27 according to average risk group (AR) 1, 10 according to AR group 2 and 4 received a very high risk protocol. The majority of the allergic reactions were classified as grade 2 and 3 according to the WHO classification; we did not observe grade 4 allergic reactions. Allergy could occur in all treatment phases (induction: 27%, consolidation: 20%, maintenance: 8%), with predominance during reinduction (45%). 26 patients were switched to Erwinia asparaginase (Erwinase®),18 to pegylated E. Coli asparaginase (Oncaspar®). Allergic patients presented significantly higher asparaginase antibodies at the time of clinical allergic reaction, compared to non-allergic patients (mean: 5,9 105 µg/L *vs.* 2,5 103 µg/L). In most allergic cases high antibody levels correlated with low asparaginase activity. Four allergic patients presented asparaginase activity >400U/L and low antibody titers. Presumably their allergic reaction was triggered by a compound of Paronal® instead of the E. Coli asparaginase itself. On the contrary we observed 3 non-allergic patients with low asparaginase activity and high antibody levels (>105 µg/L), so called silent inactivation. Currently, these 3 patients remain in complete remission. *Conclusions*. These results show that monitoring of asparaginase levels and antibody titers during treatment of children with ALL is feasible. Unexpected observations as silent inactivation and absence of antibodies after allergic reaction show the additional value of these measurements. A prospective multicenter study will unravel the importance of these asparaginase levels, antibodies and silent inactivation in the clinical course and outcome of patients treated with asparaginase for childhood ALL and will determine cross-reactivity of antibodies after switching products. Consecutive measurement of asparagine and glutamine depletion will give insight in the ideal cut-off for asparaginase activity.

0613

ARE PEDIATRIC LEUKEMIA SURVIVORS AT RISK OF DEVELOPING A METABOLIC SYNDROME IN ADULTHOOD?

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Background. long term survivors of childhood acute leukaemia are exposed to life threatening conditions during their adult life. A higher risk of cardio vascular diseases has formerly been pointed out in this population. *Aims.* to evaluate the prevalence of metabolic syndrome (MS), as defined by the National Cholesterol Evaluation Program Adult Treatment Panel III (NCEP-ATP III) and its components in the "L.E.A"(for Leucémie de l'Enfant et de l'Adolescent") French cohort. We then tried to define risk factors associated with the development of the

MS. *Methods.* this prospective study evaluates incidence and risk factors for MS in young adult leukemia survivors included in the « L.E.A » program, a French multicentric program, built in order to evaluate the long term health status, the quality of life and socio-economical status of childhood leukaemia survivors treated since 1980. During the years 2007 and 2008, assessment of MS and its components was systematically offered to all adults with a new L.E.A. health status evaluation. Metabolic syndrome was defined according to the 2005 revised NCEP ATPIII definition. Informed consent was obtained from all participants. *Results.* Out of 184 patients included in the study (mean age: 22.7 years, mean follow-up: 14.5 years), the overall frequency of the MS was 9.24% (CI: 5.47; 14.38), respectively 8.42 and 10.11%, for men and women. In a multivariate analysis, we found that a higher frequency of MS was significantly associated with total body irradiation (TBI) conditioning regimen before haematopoietic stem cell transplantation (OR: 3.88, CI: 1.13-13.32, P=0.03) compared with non TBI exposed subjects. The use of TBI was associated with a higher rate of dyslipemia (hypertriglyceridaemia, OR: 4.47, CI: 1.6-12, P=0.01, and low HDL-cholesterol, OR: 2.55, CI: 1.1-5.7, P=0.02) and elevated fasting glucose (OR: 6.11, CI: 1.11-33.47, P=0.04). By contrast, we did not find any impact of TBI on waist circumference or blood pressure. The use of cranial irradiation was not a significant risk factor, contrasting with several studies previously reported. Interestingly, MS was associated in univariate analysis with other late endocrine complications of childhood leukaemia: MS was more frequent among patients suffering from hypogonadism (23.53% *vs.* 6.00%, P=0.001), and hypothyroidism (23.53% *vs.* 7.78% out of patients without hypothyroidism, P=0.06). There was no significant association between MS and growth deficiency. *Conclusions.* MS is frequent among childhood leukaemia survivors. We have shown for the first time that TBI is a major factor involved in the genesis of the MS. The mechanisms by which these results could be explained remain to be explored.

0614

CLOFARABINE IN CLINICAL PRACTICE: THE UNITED KINGDOM EXPERIENCE IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. Clofarabine, a second-generation purine nucleoside analogue is licensed for the treatment of multiply relapsed pediatric acute lymphoblastic leukemia (ALL). Early phase trials suggest it is effective and well tolerated when combined with other cytotoxic drugs. Combination regimes are increasingly prescribed for the treatment of resistant cases. *Aims.* This study aims to draw together the United Kingdom (UK) experience of clofarabine use for the treatment of pediatric ALL. *Methods.* All UK centers that had used clofarabine for the treatment of pediatric ALL outside the context of a clinical trial were approached to offer participation in the study. *Results*. Of the 11 centers identified, 9 provided information on 23 patients who had received clofarabine. Median age was 6 years, including 5 infants. The group included 18 B-lineage leukemias, 4 T-lineage leukemias and 1 mixed lineage leukemia. Six patients had adverse cytogenetics (5 MLL rearrangements, 1 t(9:22). Patients had received a median of 2 prior treatments (Range 1-4) and 5 patients had undergone previous hematopoietic stem cell transplantation (HSCT). Five patients received single agent clofarabine, whilst 18 received combination treatment with cyclophosphamide and etoposide using a range of schedules. Clofarabine, both as a single agent and in combination regimes, was well tolerated with no treatment related deaths. Most frequent adverse events were febrile neutropenia (65%), mucositis (26%) and diarrhea (17%). Several severe infections occurred including 2 invasive fungal infections and 2 viral infections (CMV reactivation and astrovirus diarrhea). Overall response rate (ORR) was 61%; 12 achieved complete remission (CR) and 2 partial remission (PR). ORR was higher in combination regimens compared with single agent use (67% *vs.* 40%). Responses were seen in both B- and T- lineage leukemias; ORR 61% (9CR, 2PR) and 75% (3CR) respectively. There were responses in patients with adverse cytogenetics, including MLL rearrangements and t(9:22). Responses were seen in all age groups including those less than one year of age (ORR 60%, 3CR). Response rate was inversely proportional to the number of prior treatments, with the highest ORR in those who had only received 1 prior treatment (ORR 86% 6 CR). Of the 12 patients who achieved a CR, 11 went on to receive HSCT. There were 2 deaths due to transplant related mortality. Of the remaining 9 patients, 2 have relapsed, whilst 7 remain in remission with a median follow up of 12 months. *Summary/Conclusions.* Clofarabine is being increasingly used in clinical practice in the UK in the treatment of pediatric refractory ALL, Our data suggest that it is most effective when given in combination with other cytotoxics and in first relapse and can lead to durable remissions. This study reports on the use of clofarabine in infants and shows good response rates. In contrast to previous studies, these data demonstrate a high response rate in T-lineage ALL. The associated incidence of fungal and viral infections would support the use of appropriate prophylaxis

0615

UTILITY OF SINGLE TUBE, SIX COLOUR FLOW MEASUREMENT OF MRD IN CHILDHOOD B-CELL PRECURSOR ALL DURING THE FIRST MONTH OF TREATMENT ON UKALL 2003

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Background. Clearance of minimal residual disease (MRD) as assessed by PCR of antigen receptor genes is now established as the strongest independent prognostic factor in children receiving identical treatment for ALL. Flow cytometric measurement of MRD is quicker and cheaper than PCR but concerns remain over applicability, change in antigen density and relative insensitivity, particularly when four colour approaches are used. We have examined which of three 6 colour combinations provides optimal applicability stability and sensitivity for use in a single tube method. *Aims.* 1) assess utility of CD58, CD34 and CD123 when added to a common 5 marker combination CD19, CD20, CD38, CD45, CD10. 2) compare utility of the CD58 six colour tube with a re-gated four colour format (CD19/CD45/CD20/CD38), chosen to be minimally affected by steroid therapy. *Methods.* Diagnostic marrow from 52 unselected children treated consecutively for BCP ALL was screened for leukaemia associated immunophenotypes (LAIPs) defined as "empty spaces"in normal controls. LAIPS were then used to measure MRD in marrow at Day8, Day15 and Day28 of ALL 2003. Analysis was performed on a FACS Canto II using BD FACSDiva software. A minimum of 70,000 events were analysed to define a LAIP at diagnosis and 500,000 analysed at follow-up (Figure 1) .

Diagram 1: The sequential gating strategy for the 6-colour panel. Using normal bone marrow as an example, optimal separation of B cells (P1) is achieved using Side Scatter *vs.* CD19 gating. Immature B cells (P2) are separated from mature B cells (P3) using CD45 *vs.* CD19 gating. The P2 cluster was then gated for the 3 leukaemia-associated immunophenotypes (LAIPs). The P4 gates highlight the relative positions that the MRD clusters should fall into for each LAIP, termed 'MRD space'. The mature B cells (P3) are also gated for each LAIP for confirmation of maturity.

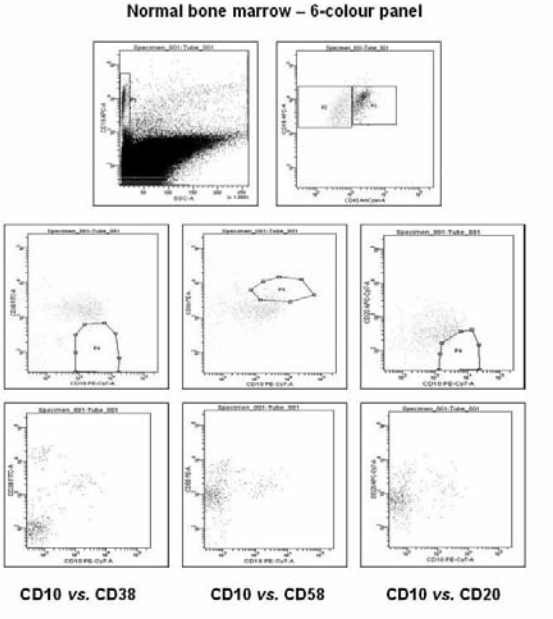

Diagram 2: The sequential gating strategy for the 4-colour panel. Using normal bone marrow as an example, optimal separation of B cells (P2) is achieved using Forward *vs.* Side Scatter, then Side Scatter *vs.* CD19 gating. The P2 cluster was then gated against the 3 LAIPs. The P3 gates highlight the relative positions that the MRD clusters should fall into for each LAIP, termed 'MRD space'.

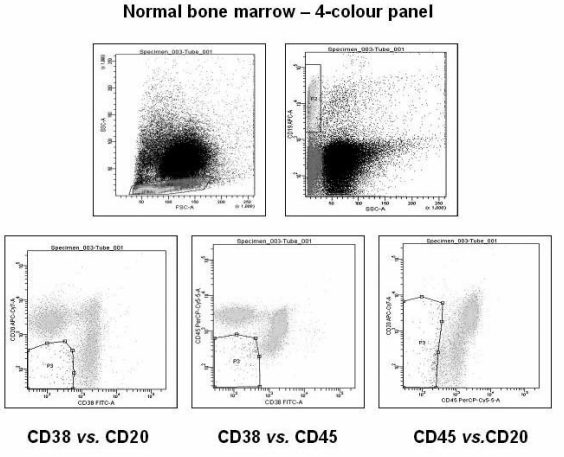

Figure 1.

Results. Applicability at diagnosis. The three six colour combinations were applicable to all patients tested - a diagnostic LAIP was defined in 52/52 screened with the CD58 combination, 36/36 screened with the CD34 combination and 31/31 screened with CD123 combination. Concordance of MRD results during the first month of treatment. 93 samples from 37 patients were analysed by multiple 6 colour marker sets (77 from 31 patients were analysed by all three). 54 of these samples were MRD positive (> 0.01%). All results were concordant between markers (<0.5 log difference). Changes in antigen density with treatment in more than 25% of cases. CD58 (41% of patients), CD34 (42%), CD10 (48%) became dimmer and CD45 (25%) became brighter. CD19 was not affected by treatment in any patient. Comparison of results of six and four colour strategies. 45 cases were screened by re-gating with 4 colours. No LAIP was defined at diagnosis in 9. In the remaining 36 cases, results of 4 and 6 colour analysis of 84 samples from 27 patients were concordant all time points (<0.5 log difference). Discordant results were seen in 22 samples from 9 patients - of particular note 8 of 9 patients had positive 4 colour results but negative six colour analysis at day 28, suggesting that four colour analysis may be cofounded by lymphoid regeneration. *Summary/Conclusions.* A single six colour combination CD19/CD45/CD20/CD38/CD10/CD58 is a widely applicable, stable marker of MRD early in therapy for BCP ALL. There is no gain in applicability from inclusion of tubes containing either CD34 or 123. Applicability, sensitivity and specificity are superior to four colour flow. At €12 the consumable cost of this single tube method is 50 times less than that of PCR of antigen receptor genes.

0616

CLINICAL OUTCOME AND MONITORING OF MINIMAL RESIDUAL DISEASE (MRD) IN PATIENTS WITH THE MLL-ENL GENETICALLY DEFINED ACUTE LYMPHOBLASTIC LEUKEMIA SUBTYPE

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Background. MLL-ENL fusion represents one of the most frequent abnormalities of ALL with the mixed-lineage leukemia (MLL) gene alteration. Because of the rarity and prevalence of this alteration in infants, the outcome of MLL-ENL positive ALL in patients of the other age groups is not firmly established. *Aims.* To better define the clinical-biologic characteristics of this leukemic subtype, we analyzed 12 MLL-ENL positive ALL patients (4 children and 8 adults), consecutively diagnosed between April 1999 and June 2009. *Methods.* At diagnosis MLL-ENL was detected by a multiplex RT-PCR, while we used the same individual primers and conditions as the multiplex system for the MLL-ENL RT-PCR monitoring of MRD, at a sensitive level of 10^{-4}. Ig/TCR gene rearrangements were detected at diagnosis and patient specific primers were designed to obtain at least two sensitive markers (^{3} 1×10^{-4}) and used to PCR-MRD. *Results.* The MLL-ENL fusion was identified in 4 (2.6%), 8 (0.6%) and 0 (0 %) of the 150 pediatric, 1215 adult and 70 elderly ALL tested patients, respectively. Four were females, 8 males. Median age was 26 years (range: 0.2-59 years). Eight patients had a WBC count $>50\times10^9$/L (median WBC = 74.5×10^9/L; range 2.9 - 707.0×10^9/L). A T-immunophenotype was detected in 3 of the 10 evaluable cases, while the 7 remaining had a B-precursor ALL (pro-B = 3; pre-pre B = 3 and pre-B = 1). Eleven out of twelve patients (92%) achieved CR; one died of infection. At 48 months 73.3% of patients is projected to be survivor and 66.7% to be event free survivor. At response evaluation, two of the 11 CR patients tested MLL-ENL negative and six positive. However, the MLL-ENL status did not correlate with outcome. In fact, the three relapses occurred in both the two RT-PCR negative cases and in one of the 6 positive patients, while the remaining 5 RT-PCR positive cases were in CR at 8, 9, 3, 3 and 5 years, respectively. In addition, MLL-ENL expression, not preceding a relapse, was detected several times during the follow-up of 5 long-survivors. In 4 cases (three children and 1 adult) therapeutic response was also determined by the parallel assessment of Ig/TCR markers. All the 4 cases remained MLL-ENL positive, but the 3 children achieved a Ig/TCR negativity, becoming long-survivors at 8, 9 and 6 years, respectively. The adult persisted Ig/TCR positive and underwent an allogeneic HSCT that induced a long-term continuous CR lasting for 5 years. Once again this case showed the MLL-ENL expression for several times during the follow-up. *Summary/Conclusions.* Present data suggests that: 1) the MLL-ENL fusion identifies a rare genetically determined leukemic entity with an extremely favourable prognosis also in adult ALL; 2) the inconsistency between the clinical cure and the presence of detectable MLL-ENL transcript, both at therapeutic response evaluation and during follow-up of long-term CR patients. This latter finding suggests the presence of a MLL-ENL-expressing "pre-leukemia" stem cells, similar to what demonstrated for the AML1-ETO positive leukemia setting.

0617

OUTCOME OF CHILDREN (NON-INFANT) WITH T(4;11) POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA TREATED WITH AIEOP-LLA 2000-R2006 PROTOCOLS

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Background. Nowadays children with ALL can be cured in more than 80% of cases. Unfortunately this rate is much lower in patients bearing specific chromosomal translocations as t(9;22) and t(4;11). The latter one is detected in 1-3% of children with ALL. However 60% of infants with ALL showed the t(4;11) translocation and a poor outcome. Conversely children with more than one year of age and bearing the same chromosomal aberration are fewer than infants and has a better outcome (Pui 2002). It is still unclear the role of chemotherapy and of hematopoietic stem cell transplantation (HSCT) in children older than one year of age with t(4;11) positive ALL. *Aim.* To evaluate response to AIEOP LLA 2000-R2006 protocol strategy in children older than one year of age (non infants) with ALL bearing t(4;11) translocation. *Materials and Methods.* Non-infants with t(4;11) positive ALL treated in AIEOP ALL 2000 and in ongoing R2006 protocols were analyzed. Patients stratification was based on prednisone response (at day 8 with blast count less than 1000/mmc), resistance (day 33) and detection of

minimal residual disease (MRD) performed at day 33 and at day 78. Children with t(4;11) were allocated to high risk treatment and eligible to a matched familiar donor (MFD) HSCT and to a matched unrelated donor (MUD) if prednisone poor responder (PPR). *Results.* We here present results of 20 children with t(4;11) positive ALL diagnosed and treated with AIEOP strategy between September 2000 and December 2007. The median age was 8 years. Eleven cases were male. The central nervous system (CNS) was involved in 2 cases. One child died before starting treatment and another one during induction (early death for infection). Six out 19 cases resulted PPR (31.5%). MRD analyses showed these results. 6 cases as HR; 11 cases as MR (4 patients were studied with one marker); one case had no evaluable markers. Relapse occurred in 8 cases (42%): 4 after front-line treatment (1 presented as AML); 4 after MFD-HSCT (1 singenic). Death occurred in 8 children who entered in the protocol: one during induction; two transplant related; five cases died of disease after relapse. Ten cases are alive in complete remission: 6 after front-line treatment; 2 after MFD HSCT; 4 after MUD HSCT. Event free and Overall survival rates at 2 years are 46.7% (SE 11.8) and 54.7% (SE 12.1), respectively. *Conclusions.* Our experience confirm that children older than one year of age with a t(4;11) positive ALL have showed an expected better outcome than infants. Moreover, However relapse rate is high, although they underwent a HSCT in first remission or showed an MRD profile as MR. Extended biological studies are needed to identify new target for a tailored-treatment in order to eradicate the disease and facilitate results of HSCT.

0618

PRESENTATION AND OUTCOME OF CHILDREN WITH DOWN SYNDROME AND ACUTE LYMPHOBLASTIC LEUKEMIA IN THE EORTC CHILDREN'S LEUKEMIA GROUP PROTOCOL 58951

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Background. Children with Down syndrome (DS) are at increased risk of developing acute lymphoblastic leukemia (ALL). Previous studies indicate a worse outcome for children with DS-ALL when compared to similarly treated children with ALL without DS (non-DS). This poorer outcome is most likely due to a lower rate of remission induction and more treatment-related toxicities. Objectives. Examination of the characteristics and outcome of DS-ALL-patients and comparison of their survival with non-DS-ALL-children. *Methods.* Exhaustive retrospective analysis of the presenting features, treatment and outcome in DS-patients with ALL treated with the EORTC Children's Leukemia Group protocol 58951. An informed consent was obtained for all patients. Results. 35 DS-ALL-patients and 1554 non-DS-patients with B-cell precursor ALL were compared. There were no T-cell ALL cases amongst DS-patients and no DS-ALL-infants. DS-patients had less often splenomegaly, higher hemoglobin and lower platelet counts. There were no adverse prognostic translocations, i.e. t(9;22) and t(4;11); and favorable cytogenetic aberrations, i.e. hyperdiploidy with >50 chromosomes and t(12;21) were less often present in the DS-cohort. DS-patients received less methotrexate and they were not included in the high risk groups (AR2 and VHR). DS-patients had inferior 5-year EFS (73.7% *vs*. 84.1%, P=0.043) and 5-year OS (81.9% *vs.* 91.5%, P=0.007), as compared to non-DS-patients. This worse outcome resulted from an increased induction failure rate (5.7% *vs.* 0.1%), mainly due to infectious deaths, and higher relapse rate (17.1% *vs.* 11%). Children with DS more often displayed severe treatment-related toxicities, especially mucositis in all treatment phases and hyperglycemia in the corticosteroid and asparaginase containing blocks. There was a tendency towards more infectious episodes in the DS group. Conclusions. Inclusion of children with DS in phase III study protocols has increased overtime and survival has improved. However, the outcome of children with DS and ALL remains worse than in ALL patients without DS because of higher risk of induction failure, mainly due to infectious deaths, and relapse. These findings indicate that we have to face the difficult challenge to increase the efficiency of the treatment with that of preventing the severe toxicities to which this particular population is oversensitive.

0619

COMPARING EMOTIONAL/BEHAVIORAL PROBLEMS BETWEEN CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA AND HEALTHY PEERS

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Background. Survival rate for so many childhood malignancies has improved during the last decade by the introduction of new treatment modalities and chemotherapeutic agents.1 For childhood Acute lymphoblastic Leukemia (ALL), which is considered as one of the prevalent malignancies in children (~%26 of overall malignancies), the long-term survival rate has reached to more than 80%in 1-10 years old children and a majority of them are actually cured.2 However, despite this great achievement, the impact and burden of such malignancies on the psychosocial qualities of these children are not well emphasized and has remained a problem, which may even seem more challenging in future. Despite great achievements in treating acute lymphoblastic leukemia in children, its burden on the psychosocial status of patients is not well understood yet. *Aims.* This study aims to determine the effect of childhood ALL on emotion, behavior and attention of patients in comparison with normal peers. *Methods.* We enrolled one hundred 6-12 years old children with ALL and 100 healthy peers as control, matched for age and gender. Mean age in the case and control group were 8.97±1.74 and 8.74±1.74, respectively. We used the Child Behavior Checklist (CBCL) from Achenbach System of empirically Based Assessment (ASEBA) to evaluate the study group. The questionnaires were completed by the child's caregiver under direct supervision of a psychologist. *Results.* There were no significant differences between the groups for age, gender and level of study at school, but this was not true for the caregivers relation and father's and mother's educational level. There existed no significant difference for school performance, but ALL group had a significant failure in group activity and social relations. Total competence was also meaningfully disturbed for ALL cases. Thought problems, attention problems, aggressive behavior, externalization, attention deficit/hyperactivity, conduct and oppositional defiant problems were more prevalent in normal children. Somatic problems were more common in ALL children. *Conclusions.* Our findings suggest that less behavioral and emotional problem among ALL children is due to better support and care from parents. Unique findings of this study emphasize the importance of more research on the psychosocial status of children with cancer.

0620

EFFICACY AND FEASIBILITY OF NELARABINE SAVAGE THERAPY IN ADULT RELAPSED OR REFRACTORY T CELL ACUTE LYMPHOBLASTIC LEUKEMIA (T-ALL) OR LYMPHOBLASTIC LYMPHOMA (T-LBL): A SINGLE-CENTER EXPERIENCE

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Background. T-ALL represents 15% of childhood and 25% of adult ALL. Despite a cure rate of almost 80% in children and adolescents, adult ALL remains a difficult disease to cure, due to a high risk of relapse. Effective treatment of relapsed acute T-ALL is limited with a low CR rate, a high treatment-related mortality, and a very low prolonged disease-free survival. Nelarabine is a pro-drug of ara-G, approved by the FDA for the treatment of T-ALL and T-LBL that have not responded to or has relapsed after treatment with at least 2 chemotherapy regimens. Similar to other nucleoside analogues, Nelarabine acts by inhibiting DNA synthesis and inducing apoptosis in malignant cells. *Aims.* of the study. To evaluate safety profile and efficacy of Nelarabine treatment as savage therapy in 9 adult relapsed or refractory T-ALL or T-LBL. *Patients and Methods.* After obtaining an informed consent, nine patients (median age 31 years, range 19-37, M/F= 9/0) affected by T-ALL (N=6) and T-LBL (N=3) received a savage therapy with Nelarabine (median cycle=1, range 1-3). Nelarabine was administered at standard adult dosage (1500 mg/sqm on days 1, 3 and 5, every 21). Seven patients were relapsed after two previous chemtotherapy regimens, including allogeneic bone marrow transplantation; the remaining two patients were primary resistant to standard induction treatment. *Results.* Five out of nine patients obtained a complete morphological remission (4 T-ALL patients and 1 T-LBL patient), whereas

a partial remission was documented in two cases, with an overall response rate of 78%. Median duration of complete response was 6 weeks (range 3-6 weeks). Nelarabine was well tolerated, and no significant adverse events were registered. Extra-hematological neurological toxicity, not clearly related to the drug, occurred in two cases, determining, in one patient a complete and irreversible paraplegia, and in the second one a condition of mental confusion (grade III), which resolved after few days. Conclusions. In our experience Nelarabine was successfully administered in such a high risk patients population. The drug showed a relevant efficacy and a good safety profile. Acknowledgments. This work was supported by European LeukemiaNet, AIL, AIRC, Fondazione Del Monte di Bologna e Ravenna, FIRB 2006, PRIN 2008, Ateneo RFO grants, Project of integreted program (PIO), Programma di Ricerca Regione - Università 2007 - 2009

0621

SAFETY AND TOLERABILITY OF INTRATHECAL LIPOSOMAL CYTARABINE AS CNS PROPHYLAXIS IN PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. Central nervous system (CNS) recurrence in acute lymphoblastic leukaemia (ALL) occurs in up to 15% of patients and depends on the intensity of front line systemic and CNS directed therapy and subtype of ALL. Meningeal recurrence -which is generally associated with a systemic relapse- not only adds significant morbidity for the individual patient but is also frequently associated with poor outcome. Liposomal Cytarabine (DepoCyte) is a sustained release formulation of Ara-C with a more homogeneous distribution throughout the neuroaxis and a prolonged half life maintaining cytotoxic concentrations in the CSF for more than 14 days. *Aims.* The purpose of our study was to evaluate the efficacy and safety of a slow-release liposomal formulation of cytarabine for intrathecal (IT) meningeal prophylaxis in patients suffering from ALL. *Patients and Results.* From 7/2004 to 2/2010 31 patients aged 20 to 68 years (median= 35) were preventively treated with a total of 75 (range: 1-6) single doses containing 50 mg of liposomal cytarabine on a compassionate use basis. Diagnoses consisted of BCR-ABL positive Pro-B-ALL (n=6), c-ALL/Burkitt lymphoma (n=2), T-ALL (n= 11), and Pro-B-ALL (n=12). All patients were treated according to the risk adapted German Multicenter Protocol/ GMALL 07/2003 including 24 Gy irradiation to the neurocranium as constituent component of CNS directed therapy. Six patients expressed the bcr-abl fusion oncoprotein and were concomitantly treated with imatinib mesylate as outlined in the study protocol. All patients received dexamethason for 3-5 days in order to prevent chemical arachnoiditis. Except for headache grade 2 in one patient no specific toxicity attributable to IT liposomal cytarabine application was noted. So far, after a median observation period of 12 months (range:1-63) 14 patients died, 10 of disease recurrence, two of GVHD after allogeneic related or unrelated stem cell transplantation, one of pulmonary artery embolism, and one of sepsis. 17 patients are in complete (n=15) or partial (n=2) remission, one after successful salvage therapy of first relapse and 8 after myeloablative allogeneic stem cell transplantation. Only one patient experienced a combined medullary - leptomeningeal disease recurrence 6 months after primary diagnosis and finally succumbed to his disease. None of the surviving ALL patients developed neurological symptoms or long term neurological side effects. *Conclusions.* IT liposomal cytarabine therapy at a dose of 50 mg with concomitant dexamethasone appears to be feasible and well tolerated. However, since all patients received concurrent systemic chemotherapy and CNS directed irradiation the efficacy of liposomal cytarabine cannot be assessed separately. Ongoing studies will further define the exact schedule for IT prophylaxis in adult patients with ALL.

0622

NO BLASTS AT DAY 8 IS BETTER THAN MORE IN CORTICO-SENSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. Acute lymphoblastic leukemia (ALL) had a good prognostic in children and young adults. However, some parameters were predictive of a worse outcome. We analysed in this study the impact of persistent blasts at day 8 of pre-phase in cortico-sensitives patients with ALL. *Patients and Methods.* Between January 2000 and December 2007, we retrospectively analysed the data of 134 children and young adults treated according the EORTC 58951 protocol. Cortico-sensitivity was considered if peripheral blast count is less than 1000/mm^3 at day 8 of prephase. The group of cortico-sensitives ALL was subdivided on two subgroups: the first with no blasts at day 8 and the second with more. We analysed the impact of cortico-sensitivity on overall survive (OS), event free survive (EFS) and disease free survive (DFS). *Results.* One hundred one child and 33 young adults were treated according to the EORTC 58951 protocol. Median age was 11 years (1 to 28 years). Sex ratio was 1.73.The patients were stratified in low risk (3 patients), average risk (96 patients) and high risk (35 patients). One hundred eleven patients (83%) were cortico-sensitive and eighty six patients had no blast (77% of cortico-sensitive patients) at day 8 of pre-phase. Complete response was obtained in 87% with no difference in different groups of cortico-sensitivity. At 68 months of follow-up, OS, EFS and DFS were respectively 65%, 60% and 75% for cortico-sensitive ALL and 22%, 25% and 49% for cortico-resistant ALL, the difference was significant with respectively P=0.002, P=0.04 and P=0.09. In cortico-sensitive patients, DFS was higher in the subgroup with no blasts: 82% *vs.* 57% (P=0.01), but no difference concerning OS (69% *vs.* 46%, P=0.46) and EFS (63% *vs.* 48% P=0.36). *Conclusions.* Cortico-sensitivity is a one of several parameters that predict good outcome in ALL. Moreover, our study showed that no blasts in peripheral blood smear at day 8 of prephase can be considered a good first marker of early blastic clearance and good outcome. Larger studies were needed to confirm this result.

0623

MRD STATUS AT THE END OF ALL-MB 2008 PROTOCOL REMISSION INDUCTION COULD BE PREDICTED BY EARLY BLAST REDUCTION

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Background. Minimal residual disease (MRD) status after remission induction is a strong prognostic factor in childhood acute lymphoblastic leukemia (ALL). Aim. To evaluate predictive impact of different clinical and laboratory parameters on MRD status after remission induction of ALL-MB 2008 protocol in children with ALL. *Methods.* From March 2008 to December 2009 56 ALL patients were enrolled onto ALL-MB 2008 trial in our institution. Among them 54 patients were included in present study based on availability of end-induction (day 36) MRD data. Study design was partially based on previously published data for AIEOP-BFM-ALL 2000 protocol [R. Ratei *et al.*, Leukemia, 2008]. MRD on day 15 (MRDd15) and day 36 (MRDd36) was assessed by 6-9-color flow cytometry (FC). MRDd36 results were categorized as "positive"or "negative". Predictive impact of initial parameters such as age, gender, CNS involvement, spleen size, initial WBC count, immunophenotype, presence of ETV6-RUNX1 fusion gene was analyzed. We also investigated predictive value of early response parameters such as absolute blast count in peripheral blood on day 8 (BCPBd8), blast percentage in bone marrow on day 15 (BPBMd15) both assessed by cytology and MRDd15. ROC curve analysis was performed, areas under curves (AUC) were compared and threshold levels (TL) were defined. Variables were categorized as "high"and "low"in respect to TL. Univariate and multivariate odds ratios (OR) with 95% confidential intervals (CI) were estimated in multiple logistic regression model with a step-by-step identification of significant parameters. Positive and negative predictive values (PPV, NPV), sensitivity, specificity and overall correct prediction (OCP) were calculated. *Results and discussion.* Among all initial parameters difference between day 36 MRD-positive and MRD-negative patients was observed only for age (P=0.03). Hence age was added to early response parameters for ROC curve analysis. By AUC comparison MRDd15 (AUC=0.85) differed significantly from BPBMd15 (AUC=0.72, P=0.049), age (AUC=0.68, P=0.02) and BCPBd8 (AUC=0.64, P=0.004). TLs were defined as 0.160% for MRDd15, 0.5% for BPBMd15, 3 years for age and 38 blasts/_l for BCPBd8. Patients with high MRDd15, BPBMd15, BCPBd8 and older than 3 years have significantly higher chance to be MRD-positive after remission induction. MRDd15 and age remained significantly contributing variables (P=0.003 and P=0.040 respectively) to the regression model with an overall correct prediction rate of 77.36%. Diagnostic performance tests are shown in table. Thus among all analyzed parameters high MRD level on day

15 is the strongest predictor of MRD-positivity after remission induction in our series of childhood ALL patients treated by ALL-MB 2008 protocol. Despite insignificant difference between day 36 MRD-positive and MRD-negative patients (P=0.056) BCPBd8 showed appropriate OCP, better than age and BPBMd15. Conclusion. MRD status in childhood ALL after remission induction of ALL-MB 2008 protocol could be predicted by early blast reduction parameters and age while MRD level on day 15 is the strongest single predictor.

Table 1. Diagnostic performance tests.

	OR	95% CI	p	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	OCP (%)
Univariate analysis								
MRDd15	29.08	3.46-244.47	.002	95.45	58.06	61.76	94.74	73.59
BPBMd15	6.33	1.56-25.71	.010	86.36	50.00	54.29	84.21	64.81
Age	4.97	1.47-16.86	.010	77.27	59.38	56.67	79.17	66.67
BCPBd8	5.16	1.56-17.02	.007	59.09	78.13	65.00	73.53	70.37
Multivariate analysis								
MRDd15	25.53	2.90-224.50	.003	-	-	-	-	-
Age	4.43	1.07-18.34	.040	-	-	-	-	-
Regression model	11.11	3.12-39.65	.0001	72.73	80.65	72.73	80.65	77.36

0624

HIGH DOSE METHOTREXATE TREATMENTS IN CHILDHOOD LEUKEMIA - COMPARISON OF BFM 1995 AND 2002 PROTOCOLS

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Methotrexat (MTX) is a widely used antifolate cytostatic agent for the treatment of different childhood malignancies, however the exact dose and mode of application is not clearly defined. The aim of our study was to compare high dose MTX treatments by analysing the pharmacokinetic parameters and toxicity after the therapies. We investigated children with acute lymphoblastic leukemia (ALL) treated according to the ALL-BFM-1995 and 2002 protocol at the 2nd Department of Paediatrics at Semmelweis University between 1998-2006. *Patients and Methods.* 43 children were treated with 5 g/m^2/24 h MTX and 39 children with 2 g/m^2/24 h MTX according to the protocols. The mean age of the patients was 7.1 years (0.5-16.7). Totally 283 MTX infusions were analysed. Serum MTX and 7-OH-MTX levels were measured with HPLC at 24, 36, 48 hours, while the MTX concentration in the CSF was determined at 24 hours after the start of MTX infusion. Considering the toxicity of the treatments we measured the serum ALAT, ASAT, bilirubin, creatinine, protein levels before therapy and one day, two days and one week after treatment. *Results.* Mean MTX level at 24. hours and 7-OH-MTX level at 36. hours were significantly lower after 2 g/m^2 courses than after 5 g/m^2 courses (MTX2: 29.7+/-17.4 μmol/L; MTX5: 89.5+/-55.0 μmol/l; 7-OH-MTX: 4.1+/-1.7 μmol/L and 7.6+/-5.2 μmol/L; P<0.05). In more than 50% of the cases with 2 g/m^2 MTX serum levels were below 30 μmol/L (therapeutic level). Comparing CSF MTX concentrations we did not find significant difference between the two doses (after 2g/m^2: 15.1+/-41.7 μmol/l; after 5g/m^2: 23.3+/-41.5 μmol/l). In children who received 5 g/m^2 MTX significantly more cases of hepatotoxicity, trombocytopenia, mucositis occurred, however these side effects were mild and reversible. 7-OH-MTX levels showed closer correlation with the toxicity parameters than MTX (P=0.0004). *Conclusion.* 5 g/m^2 MTX resulted more reliable therapeutic serum levels with slightly more toxicity. 7-OH-MTX measurements might be more useful than MTX levels to detect toxicity. However, further randomised studies are necessary to determine the optimal dose of MTX.

0625

MRD - BASED RISK STRATIFICATION IN CHILDHOOD B-ALL USING RQ-PCR OF IMMUNOGLOBULIN (IG) AND T-CELL RECEPTOR (TCR) GENE REARRANGEMENTS - A SINGLE CENTER EXPERIENCE

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Background. Several studies have shown that MRD quantification using RQ-PCR for detection of Ig/TCR gene rearrangements has prognostic value in childhood ALL. Different clinical trials already incorporate MRD-based risk stratification. *Aims.* We report RQ-PCR-based MRD results obtained on d32 of induction therapy in 71 childhood B-ALL patients (pts) treated according to the DFCI ALL-05 protocol. We compared the sensitivity levels and RQ-PCR-based MRD data obtained by cloning the Ig/TCR rearrangements into plasmids (according to DFCI-ALL-05 protocol) and using the EuroMRD guidelines. *Methods.* Bone marrow (BM) samples were obtained at diagnosis from 71 childhood B-ALL pts (30 females, 41 males, median age 5.7, 1.1 to 16.4 yo) from March 2007 to December 2009. Risk group stratification according to age, WBC, immunophenotype, CNS leukemia and cytogenetic at diagnosis showed 55/71 (77%) standard, 15/71 (21%) high and 1/71 (1%) pts at very high risk of relapse. Intensified therapy was administered to patients in the very high-risk group or with MRD>0.001 at d32 BM sample. Clonal Ig/TCR gene rearrangements were identified according to the BIOMED-1/2 strategies. After sequencing (ABI 3130) at least one allele-specific oligonucleotide (ASO) was designed for each patient. Experimental set-up of RQ-PCR (7900-HT) and interpretation of MRD data was done according to the EuroMRD guidelines and based in standard curves obtained from serial dilutions of diagnostic clonal DNA. Standard curves obtained from plasmid dilutions containing the target DNA were compared to curves obtained from serial dilutions of diagnostic clonal DNA using 10 Ig/TCR rearrangements: VH2-JH1, VH4-JH2, VH3-JH4, VH4-JH4, VH3-JH5, VH2-JH6, Vk7-Kde, Vg5-Jg1.3/2.3, Vb30-Jb2, Vd2-Dd3. *Results.* In 71 patients, 254 rearrangements were sequenced and 129 used as ASOs: 88 IGH, 18 incomplete TCRD, 8 TCRB, 8 IGK and 7 TCRG rearrangements. The majority (122/129) of targets had a sensitivity ≥10^{-4}; in 2/129 the sensitivity was <10^{-3} precluding its use. In 57% (74/129) a quantitative range (QR, reproducible sensitivity) of at least 10-4 was obtained. Globally, MRD-based stratification was feasible in 67/71 patients: 1/71 had resistant disease, 1 had no BM sample at d32 and 2/71 had no sensitive target. In 43/67 pts, MRD was quantified with two (34 pts) or three (7 pts) targets. At d32, 32/67 (48%) patients had undetectable MRD, 21/67 (31%) had positive non-quantifiable disease, 6/67 (9%) had MRD levels <0,001 and 12% (8/67) had MRD level >0.001 and were re-stratified into the very high-risk group. RQ-PCR-based MRD levels obtained with standard curves generated for ten different targets and using either plasmid or clonal diagnostic DNA dilutions showed highly concordant results (P<0,05, Pearson correlation r=0,894). All targets had a sensitivity of 10^{-4}, according to EuroMRD guidelines. Using the DFCI-05 protocol strategy 9/10 targets reached a sensitivity of 10^{-4}. *Conclusions.* Our RQ-PCR strategy based on the EuroMRD guidelines allowed therapy intensification in 8/67 (12%) B-ALL pediatric patients treated according to the DFCI-ALL-05 protocol. The use of a more simple RQ-PCR method based in serial dilutions of clonal diagnostic DNA, minimizing the risk of PCR contamination enables a highly sensitive, reproducible and fast MRD quantification in precursor-B- ALL patients.

Acute myeloid leukemia - Biology 3

0626

NM23-H1 PROMOTES AML CELL SURVIVAL VIA CROSS TALK BETWEEN THE MORE IMMATURE AND MATURE COMPONENTS OF THE CLONE

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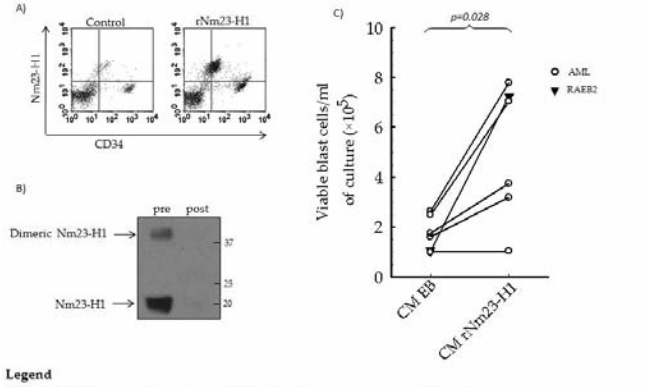
Background. Nm23-H1 protein is elevated in the serum of AML patients, and its levels correlate with the white cell count at diagnosis and, therefore, prognosis. Recent data from Okabe-Kado and colleagues (2009) suggests that this association is causal and that Nm23-H1 acts as an AML survival factor. However, the role of Nm23-H1 in exacerbating the tumour burden in AML is poorly understood. *Aim.* The aim of this study was to delineate whether the effect of Nm23-H1 on AML cells is mediated via actions against the immature cells or their more mature progeny. *Methods.* Primary AML cells from 18 patients were cultured with and without 2 μg/mL rNm23-H1 in serum-free conditions. Analysis of cell surface Nm23-H1 binding, as determined by flow cytometry, revealed preferential binding to the more mature component of the clone. Cells from 5 AML patients and 1 RAEB2 patient were consequently sorted in to immature ($CD34^{+ve}$ and/or $CD117^{+ve}$) and more mature ($CD34^{-ve}$ and/or $CD117^{-ve}$) fractions. The $CD34^{-ve}/CD117^{-ve}$ cells were treated with 2 μg/mL rNm23-H1 for 20 hours, after which the resulting conditioned medium was depleted of rNm23-H1 using nickel resin. Subsequently, the $CD34^{+ve}/CD117^{+ve}$ cells, which had been stored at 4°C overnight, were cultured in this conditioned medium, and cell survival was analysed after 2 days in culture. Nm23-H1 induced cytokine release from the $CD34^{-ve}/CD117^{-ve}$ cells was investigated using a luminex assay. *Results*. rNm23-H1 enhanced the survival of those AMLs that bound rNm23-H1 at the cell surface (n=11), whilst there was little effect on AMLs which did not detectably bind rNm23-H1 (n=7). The population of cells which bound rNm23-H1 were $CD34^{lo}/CD34^{-ve}$ and $CD11b^{+ve}$, indicating that the survival effect on the more immature cells was indirect. In 5 out of 6 cases, the survival of immature $CD34^{+ve}/CD117^{+ve}$ blast cells was enhanced by medium conditioned by rNm23-H1 stimulated $CD34^{-ve}/CD117^{-ve}$ cells, but not by medium conditioned by non stimulated $CD34^{-ve}/CD117^{-ve}$ cells. Analysis of the $CD34^{-ve}/CD117^{-ve}$ cell conditioned medium demonstrated that rNm23-H1 stimulated the release of multiple cytokines including IL1β, IL6, MCP-1, IL8, and VEGF. This effect was not the result of LPS contamination of rNm23-H1 as the addition of the LPS inhibitor polymyxin B to cultures had no effect on induced cytokine release.



Legend

A) Nm23-H1 binds a population of $CD34^{lo}/CD34^{-ve}$ cells to mediate AML cell survival
B) Nm23-H1 western blot of $CD34^{-ve}/CD117^{-ve}$ conditioned media pre and post rNm23-H1 depletion
C) Medium conditioned by rNm23-H1 stimulated $CD34^{-ve}/CD117^{-ve}$ cells enhances the survival of immature $CD34^{-ve}/CD117^{-ve}$ blast cells (CM EB: conditioned medium from elution buffer control treated $CD34^{-ve}/CD117^{-ve}$ cells, CM rNm23-H1: conditioned medium from rNm23-H1 $CD34^{-ve}/CD117^{-ve}$ cells)

Figure 1.

Conclusion. It has been previously shown that Nm23-H1 is highly expressed in normal immature $CD34^{+ve}$ haemopoietic cells, and that its expression decreases during haemopoietic maturation (Willems *et al.,* 1998). Thus it is likely that the elevated serum Nm23-H1 levels found in AML patients derive from the more immature components of the clone. Collectively our findings indicate that the released Nm23-H1 is sensed by the more mature malignant cells, improving their survival, and stimulating a reciprocal cytokine mediated survival signal directed at the immature cells. This novel feedback mechanism reveals that the action of Nm23-H1 in promoting AML cell survival and clonal expansion is more complex than expected. Following the demonstration that Nm23-H1 inhibits myeloid cell differentiation (Okabe-Kado *et al., 1995*), we hypothesise that this mechanism may reflect the subversion of a previously unidentified process of quorum sensing during normal haemopoiesis.

0627

A NOVEL NUP98/RARG REARRANGEMENT IN AN ACUTE MYELOID LEUKEMIA RESEMBLING PROMYELOCITIC PHENOTYPE

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Background. The NUP98 gene at 11p15 is known to be fused to more than twenty other partner genes in various hematologic malignancies. The common in all NUP98 chimeras is a transcript consisting of the 5' part of NUP98 and the 3' portion of the partner gene. Despite the high homology sequence (90%) of the RARA gene with the other members of the retinoid acid receptor family (RARB and RARG), rearrangements involving these last two genes have not been reported in human leukemias. *Aims.* To determine the genes involved in a novel t(11;12) translocation in a patient with an AML morphologically resembling an APL. Case History. A 35-year-old male referred to our department because of asthenia, mucosal bleeding, ecchymoses and fever. Blood tests showed anemia (hemoglobin 6 g/dL) and thrombocytopenia (platelet count 8×10^9/L) and white blood cell counts (12×10^9/L) with 82% blasts. Morphological and immunophenotype picture in bone marrow resembled the hypergranular subtype of acute promyelocytic leukemia (M3) of the French-American-British Classification, but lacking the PML/RARA rearrangement on molecular analysis or the t(15;17) in karyotype and FISH. The karyotype showed, however, a translocation t(11;12)(p15;q13). The patient underwent treatment with PETHEMA AML 2007 protocol based on a 3+7 schedule of chemotherapy for induction and consolidation followed by an autologous peripheral blood transplant. Six months after transplant, the patient remains alive in complete remission. *Methods.* Array-CGH fine mapping of minor or cryptic genomic imbalance was performed in bone marrow sample. Labelled DNA was hybridized with Human Genome CGH Microarray 244K (Agilent p/n G4423B-014693) containing 236,000+ coding and noncoding human sequences represented. DNA control sample extracted for the patient's lymphocytes was used as reference in match hybridizations. Arrays were scanned in an Agilent Microarray Scanner (Agilent G2565BA) and data extracted using Agilent Feature Extraction Software 9.5.3.1. The NUP98-RARG mRNA was determined on random hexamer reverse transcribed cDNA using primers to NUP98 5'-GGGCTTGGTGCAGGATTTGG-3') with the reverse RARG (exon 7: 5'-GCTGACCCTGAACCGGACCCA-3'). These primers were also used to amplify the genomic breakpoint. *Results.* We found a 1.0Mb microdeletion in 11p15 affecting NUP98 gene and 2.5Mb microdelection in 12q13 affecting RARG gene. The other entire genomic regions presented a normal copy of the DNA. To confirm the NUP98-RARG fusion, an 881bp product was specifically amplified from patient cDNA but not from the control cDNA. The genomic breakpoint product was co-amplified in the same reaction and sequenced. The NUP98-RARG fusion mRNA was predicted to encode a 862 amino acid protein of 97 KDa, that retains the N-terminal region motif of the nuclear export of poly(A)+ RNA and nuclear pore complex (NPC) structure from NUP98 fused to the entire tyrosine kinase domain of RARG. *Conclusion.* We report a novel rearrangement involving the subunit gamma of the retinoid acid receptor (RARG) and the nuclear pore complex protein NUP98, a known promiscuous gene frequently involved in AML and myelodysplastic syndromes. The morphological and immunophenotypic features resembled those in hypergranular subtype of APL.

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0628

HEMATOGONES : A NEW PROGNOSTIC FACTOR IN ACUTE MYELOBLASTIC LEUKEMIA

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Despite the identification of several baseline prognostic factors, the outcome of patients with acute myeloid leukemia (AML) is still heterogeneous. New prognostic parameters are needed to assess the risk of relapse, in particular in patients without aberrant leukemia associated phenotypes (LAPs) and in absence of fusion genes derived from chromosome translocations. Hematogones (HGs) are normal B-lymphocytes precursors described in healthy subject, increasing in several hematological and non-hematological disease and in marrow regenerative state following chemotherapy. We investigate, by flow-cytometry, the prognostic impact of HGs on leukemia free (LFS) and overall survival (OS) in 120 patients with AML in first complete remission (CR). In univariate analysis, leucocytosis, favourable cytogenetic and HGs>0,1% were associated with improved LFS and OS. Patients with positive marrow HGs in first CR have significant higher LFS (29.2 *vs.* 11.7 months : P=0.0011) and OS (31.7 *vs.* 21 months: P<0.03) than patients without. Furthermore predictive value of HGs for a better LFS is still significant, when adjusted for cytogenetics and leucocytosis in a multivariate analysis (P<0.047). The presence of HGs in first CR with a cut-off of 0.1% can be a useful tool to predict relapse and survival in acute myeloblastic leukemia.

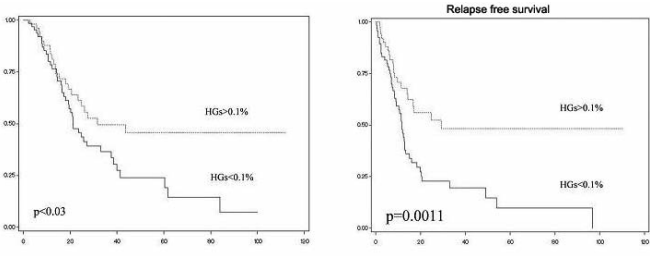

Figure 1. OS and LFS according to HGs status.

0629

FMR1NB IS THE FIRST CANCER-TESTIS ANTIGEN EXPRESSED ON THE SURFACE OF MALIGNANT CELLS

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Background. Cancer-testis (CT) antigens represent ideal candidates for tumor-specific therapy due to their exceptional tissue restriction. Unfortunately, most CT antigens are inaccessible to immediate targeted therapies due to their predominantly intracellular localization. CT antigen FMR1NB might represent a promising exception as prediction algorithms indicate a putative transmembrane topology. *Aims.* The aims of our study were the conclusive determination of the subcellular localisation of FMR1NB and to assess the possibility to target this protein on the cell surface using specific antibodies. *Methods.* We screened acute (AML) and chronic (CML) myeloid leukemia samples as well as healthy tissues for the expression of FMR1NB. Mammalian cell lines were transfected with FLAG- or GFP-tagged FMR1NB expression constructs. We evaluated the subcellular localization of FMR1NB by fractionation and confocal microscopy. Epitopes of commercial anti-FMR1NB antibodies were characterized by ELISA using overlapping peptides, and localization and expression of the protein in AML cells was evaluated by immunofluorescence stainings and Western Blot. *Results.* FMR1NB mRNA was expressed in AML cell lines and patient samples and confirmed by western blot on the protein level. Specificity was confirmed by the lack of FMR1NB expression in healthy tissues. Confocal fluorescence microscopy of GFP chimeras in mammalian cell lines, immunofluorescence staining of AML cell lines, and subcellular fractionation strongly support a membraneous localization of FMR1NB including the plasmamembrane. In contrast to results of structural prediction software our characterization of epitopes recognized by polyclonal anti-FMR1NB antibodies and the subsequent immunostaining of intact transfected and native AML cells suggest an extracellular amino-terminus of FMR1NB. Flow cytometric analysis of AML and CML cell lines as well as AML patient samples and healthy donors further confirmed a strong and specific expression of FMR1NB on leukemia samples. *Conclusions.* FMR1NB mRNA is specifically expressed in AML cells and the resulting protein localizes to the membrane compartment including the plasma membrane. A putative extracellular domain of over 100aa length of the protein can be detected using FMR1NB-specific antibodies. The membrane localization of FMR1NB is a rare exception within the CT antigen family and our data suggest that it represents an ideal target for the diagnosis and therapy of myeloid leukemias.

0630

FUNCTIONAL DEREGULATION OF NF-κB AND ABNORMAL TNFα RESPONSE IN ACUTE PROMYELOCYTIC LEUKEMIA

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Background. Acute promyelocytic leukemia (APL) accounts for approximately 10% of acute myelogenous leukemia (AML) cases, and is characterized by accumulation of abnormal promyelocytes in patient bone marrow and peripheral blood. APL is associated with balanced chromosomal translocations involving retinoic acid receptor alpha (RARA), giving rise to fusion oncoproteins referred to as X-RARA. *Aims.* As deregulation of retinoid signaling is insufficient for leukemia development, our studies aim to determine other signaling pathways involved in APL by assessing the gene expression profiles and cell biology of X-RARA. Methods and *Results.* We previously determined, using gene expression microarray analysis, common downstream targets of the variant APL fusion proteins NPM- and NuMA-RARA. We observed an over-representation of NF-κB target genes within this dataset. In these cells, a number of NF-κB target genes were commonly over-expressed. A subset of commonly deregulated genes were validated in our APL cell lines and 23 primary APL patient samples by real-time quantitative RT-PCR (RQ-PCR). 13/16 genes that were tested were significantly altered in APL compared to normal BM (n=11) P<0.05. The majority of these deregulated genes showed a progressive trend towards normal expression levels in post-treated samples. These data indicate defects in NF-κB-mediated gene expression in APL pathogenesis. We next examined NF-κB activity by assessing the expression levels of NF-κB target genes by quantitative real-time RT-PCR, in the presence and absence of TNFα. We observed that there was sustained activation of NF-_B in cells expressing NPM-RARA, as evidenced by the increased expression of NF-κB transcriptional target genes after induction by TNFα. This was also evident by sustained increases in levels of phospho-p65/NF-κB in U937-NPM-RARA cells after TNFα treatment. Western analysis of protein derived from U937-NPM-RARA and NB4 cells demonstrated over-expression of NF-κB (p65) protein, as well as its transcriptional target IκBα. The increased pool of NF-κB localized to both the cytoplasm and the nucleus in U937-NPM-RARA cells as visualized by immunofluorescent confocal microscopy. Having observed deregulated expression of downstream targets of TNFα, we sought to examine the ability of X-RARA to confer resistance to TNFα-mediated apoptosis. Our results in colony formation assays indicated that NPM-RARA expressing cells formed significantly more colonies, in the presence of 0-100 ng/mL TNFα, in a dose-dependent manner, compared to U937 control cells. Summary/*Conclusions.* These data suggested a greater ability on the part of NPM-RARA+ cells to survive and proliferate in the presence of TNFα. Our data provides the first evidence of the functional deregulation of the NF-κB-mediated signaling pathway and the TNFα response in cells expressing the variant APL fusion proteins.

0631

INCREASED RISK OF ACUTE MYELOID LEUKEMIA IN PATIENTS WITH CYP1A1 POLYMORPHISMS

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Background Acute Myeloid Leukemia (AML) is a heterogeneous group of genetically diverse hematopoietic malignancies arising from blood cell progenitors developing in the myeloid pathway or from primitive stem cells with multilineage potential. Till current knowledge it seems the development of AML probably arises through a combination of genetic

susceptibility and environmental factors most of which are not yet fully understood. Thus, very little is known related to inherited factors (genetic susceptibility of AML) that contribute to DNA damage or poor detoxification of carcinogens and chemical compounds. From experimental data DNA damage in the haematopoietic precursor cell is the essential pre requisite for the development of AML. DNA damage can be induced directly by chemical mutagens, partially metabolized carcinogens that bind directly to DNA forming DNA adducts. Chemical compounds are metabolized by a two phase detoxifying system. CYP1A1 is a a cytochrome P-450 phase-1 superfamily enzyme involved in the bioactivation of environmental pollutants (byproducts of fuel burning, lubricant oils, fossil fuel combustion, coal, vehicle exhaust, tar and cigarette smoke). The four CYP1A1 polymorphisms are: CYP1A1*2A, CYP1A1*2C, CYP1A1*3 and CYP1A1*4. Phase-2 metabolism polymorphisms (del{GSTM1} and del{GSTT1}) result in loss of enzymatic activity. The genetic component of AML etiology is likely to be polygenic and the identification of further candidate genes is essential for future studies and assessment of AML risk-related genes. Aims We determined the frequencies of carcinogen metabolism gene polymorphisms (CYP1A1, del{GSTM1} and del{GSTT1}) in a case control-study (control group and AML patients in Sao Paulo, Brazil) based on polymorphism analysis. Methods We studied 58 consecutively AML patients (29 males and 29 females, median age: 62 years, range 18 - 91 years) and 174 age and sex-matched control group (CG, 90 males and 84 females, median age: 50 years range 19-74 years). To verify if any of those genetic polymorphisms could pose a risk for AML there had been calculated three sex and age controls for each AML patient (alpha =0.05% (bicaudal), statistical power 80% beta=0.20) with an estimated odds ratio (OR) equal or less than 2. Hardy-Weinberg equilibrium was also calculated. CYP1A1 polymorphisms (CYP1A1*2A, CYP1A1*2C, CYP1A1*3 and CYP1A1*4) were assessed by a PCR-RFLP assay (Cascorbi *et al.*, 1996). Polymorphisms were categorized as wild-type, heterozygous or mutant. Del{GSTM1} and del{GSTT1} were studied using a PCR assay (Gattás *et al.*, 2000). Beta-globin gene was amplified as an internal control. Results. There were 51 *de novo* AML and 7 secondary AML. CYP1A1*2A and CYP1A1*2C polymorphisms were much more frequent in CG than AML P<0.001 and in contrast, CYP1A1*3 and CYP1A1*4 polymorphisms frequencies were significantly more frequent in AML than CG P<0.001. There were no differences found in del{GSTM1} neither del{GSTT1} between AML (P=0.999 and P=0.539). The OR for AML patients with CYP1A1*3 genotype was 2.36 (95% CI 1.2 - 4.5) and 2.38 for CYP1A1*4 (95% CI 0.8 - 6.8). Adjusted OR (for sex and age) was 2.63 for CYP1A1*3 (95% CI 1.4 - 5.1) and 2.66 for CYP1A1*4 (95% CI 0.9 - 7.8). In the multivariate analysis CYP1A1*3 polymorphism was a risk factor for AML with an OR for 3.99 (CI 1.9-8.6 95%) (Table). On the other hand CYP1A1*2A and CYP1A1*2C heterozygous polymorphisms presented a protective genotype AML risk (OR 0.54, 95% CI 0.1 - 0.6 and OR 0.23 0.1-0.5 respectively, for CYP1A1*2A and CYP1A1*2C). Conclusions. To the best of our knowledge this is one of the few studies assessing the risk of AML in individuals with xenobiotic metabolism polymorphisms. Our data supports that inherited absence of this carcinogen detoxification pathway may be an important determinant of AML. To the best of our knowledge this is the first study to show that CYP1A1*3 heterozygous genotypes increase the risk of AML. Biological effects of this genotype in AML are still unknown and require further investigation.

Table. Estiamated relative risk of AML, crude and adjusted odds radio by univariate and multivariate analysis.

Univariate analysis		Crude OR (95% CI)	Adjusted OR (95% CI)
*CYP1A1*2A*	Wild type	1.00	1.00
	Heterozygous	0.21 (0.1 – 0.4)	0.20 (0.1 – 0.4)
	Mutated	0.13 (0.0 – 1.1)	0.14 (0.0 – 1.1)
*CYP1A1*2C*	Wild type	1.00	1.00
	Heterozygous	0.23 (0.1 – 0.4)	0.19 (0.1 – 0.4)
*CYP1A1*3*	Wild type	1.00	1.00
	Heterozygous	2.36 (1.2 – 4.5)	2.63 (1.4 – 5.1)
	Mutated	0.39 (0.0 – 3.2)	0.44 (0.1 – 3.7)
*CYP1A1 *4*	Wild type	1.00	1.00
	Heterozygous	1.31 (0.7 – 2.6)	1.31 (0.7 – 2.6)
	Mutated	2.38 (0.8 – 6.8)	2.66 (0.9 – 7.8)
GSTM1	Present	1.00	1.00
	Null	1.00 (0.5 – 1.8)	0.95 (0.5 – 1.7)
GSTT1	Present	1.00	1.00
	Null	1.27 (0.6 – 2.8)	1.37 (0.6 – 3.0)
Multivariate analysis			**Adjusted OR** (95% CI)
*CYP1A1*2A*	Wild type		1.00
	Heterozygous		0.28 (0.1 – 0.6)
	Mutated		0.54 (0.1 – 5.3)
*CYP1A1*2C*	Wild type		1.00
	Heterozygous		0.23 (0.1 – 0.5)
*CYP1A1*3*	Wild type		1.00
	Heterozygous		3.99 (1.9 – 8.6)

0632

BROAD COPY NEUTRAL-LOSS OF HETEROZYGOSITY REGIONS AND RARE RECURRING COPY NUMBER ABNORMALITIES IN KARYOTYPICALLY NORMAL ACUTE MYELOID LEUKEMIA GENOMES

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Background. Approximately 40-50% of AML are karyotypically normal (KN-AML) and prognostically heterogeneous, showing various molecular alterations. Recent advances in genome-wide analysis of submicroscopic DNA segment copy number variations (CNVs) may allow the identification of novel molecular tumor-associated abnormalities (somatic copy number abnormalities, CNA). However, CNVs are also present physiologically in the normal population (germline CNVs) and can represent potential predisposition factors in disease. Loss of heterozygozity (LOH) is an allelic imbalance in polymorphic loci, resulting from genomic losses or gains leading to unequal ratios of parental alleles. *Aims.* To test the ability of the last generation of Affymetrix single nucleotide polymorphism (SNP)/CNV platform (SNP Array 6.0) to distinguish somatic tumour-associated CNAs and LOHs from germline CNVs and LOHs and to identify possible recurring genomic abnormalities. *Methods.* 19 patients have been studied, 9 females, 10 males (median age 42 years, range 25-70) using bone marrow aspirates collected at diagnosis (Dx) and at the first complete remission (R), defined as karyotypically-normal on the basis of standard metaphase cytogenetics (MC). We re-analysed them using the SNP Array 6.0 Assay kit (Affymetrix, Santa Clara, CA) and HapMap 270 DNA as reference to detect SNP and LOH. Signal intensity was analysed using Genotyping Console Version 3.0.1. We obtained quality control (QC) call rates in excess of 90% in all cases and MAPD <0.4. In 11 cases we obtained a bone marrow sample at the remission phase of comparable high quality, used to obtain a first estimate of tumor-associated CNAs. All Dx segments not overlapping for more that 30% of their size the corresponding R segments were included in a list of somatic CNAs. For each sample algorithms implemented in the GTC software provided a list of all gains or losses (DNA segments spanning multiple consecutive markers showing an increase or a decrease in copy number in comparison to a reference normal population). The number of tumor-associated CNAs were determined after comparison of matched Dx/R samples using stringent conditions able to reduce the number of false positive CNAs. Results. With the exception of a single outlier case, we observed few recurring CNAs per sample (Table 1). However, a high prevalence of CNAs in the KN-AML population was detected, thus providing new hints towards identification of cooperating mutations. In particular, our results point to the region 3p14.1-p12.3 as a target for the identification of driver mutations in AML. An extensive search of all tumour-associated CN-LOH regions > 1 Mb revealed only 3 broad regions (terminal 12Mb of 22q, terminal 27Mb of 1p and the whole chromosome 21) in three patients out of 19 (16%). All CN-LOH segments lower than 10 Mb were not tumour-associated, as shown by their presence in corresponding matched R samples. CN-LOH of the whole chromosome 21 was responsible for homozygosity of a missense mutation (R80C) of RUNX1/AML1. Conclusion. Our study confirms that a relative submicroscopic copy number stability, with low recurrence of specific CNAs and broad CN-LOH regions, characterises KN-AML.

Table 1.

# Somatic losses	0	1	2	≥3
Observed in % of KN-AML patients (n=19)	27%	27%	18%	27%
# Somatic gains	**0**	**1**	**2**	**≥3**
Observed in % of KN-AML patients (n=19)	37%	18%	18%	27%

0633

ANALYSIS OF THE CELLULAR TARGET OF SECONDARY MUTATIONS IN C/EBPA-ASSOCIATED ACUTE MYELOID LEUKEMIA

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Acute Myeloid Leukemia (AML) is a poor prognosis-associated malig-

nant condition in the hematopoietic system characterized by rapid accumulation of abnormal myeloid progenitor cells in the hematopoietic as well as extra-hematopoietic organs. Development of AML occurs by a multistep process, involving several genetic and/or epigenetic events. This is especially relevant in patients with pre-clinical stages of disease, and patients in remission, who eventually undergo relapse. These two groups of patients host pre-leukemic populations carrying the initial mutations, but lacking the final mutations leading to a fully transformed phenotype. Like the hematopoietic stem cell in normal hematopoiesis, only a sub-population of the leukemia (hereafter referred to as the Leukemia Initiating Cells (LICs)) possesses the self-renewal capacity necessary for maintaining the leukemic population. The transcription factor C/EBPalpha is crucial for late differentiation events in numerous physiological processes, including myeloid differentiation (C Nerlov 2004, M.B Schuster 2006 , H. Leroy 2005). Therefore, it is not surprising that CEBPA mutations occur in about 9 % of all AML cases (C. Nerlov 2004). Since identical CEBPA mutations are almost uniformely present at initial diagnosis and relapse (L-Y Shih 2006), these mutations are early events in leukemogenesis and hence must be present in pre-leukemic stages of disease development as well as in patients in remission before relapse. In this study, we used transgenic mice carrying one of the most common Cebpa mutations (hereafter termed Lp30) known from AML-patients. Homozygousity for this mutation results in a myeloid differentiation-block, downstream of Granulocyte Macrophage Progenitors (GMPs). This phenotype progresses into AML with full penetrance (P. Kirstetter 2008). Since the LICs in leukemic animals resemble committed myeloid progenitors, we hypothesize that the potential to acquire a malignant phenotype resides within committed myeloid progenitors (i.e. GMPs) of pre-leukemic animals and can be delineated by expression profiling of these cells. A logical extension of this hypothesis is that GMPs constitute targets for secondary mutations. As a negative control, we used another Cebpa mutation termed the Basic Region Mutation 2 (BRM2). Similar to Lp30, BRM2-homozygousity results differentiation-block, downstream of GMPs (B.T Porse 2005), albeit only with a limited progression into AML. Gene expression profiling revealed that certain molecular pathways were differentially regulated in Lp30-GMPs *vs.* BRM2-GMPs. Specifically pathways involved in signal transduction, lymphoid differentiation, cell motility and leukemic transformation were upregulated.To identify Lp30-GMPs as the direct target of secondary mutations, we compared BRM2-GMPs and Lp30-GMPs in terms of differentiation potential, self-renewal capacity and immortalization. We found that while both Lp30-GMPs and BRM2-GMPs displayed similarly impaired *in vitro* differentiation potentials, only Lp30-GMPs spontaneously immortalized into cells displaying morphologies and surface marker profiles resembling myeloid progenitors, as well as efficient short-term homing capacity. No AML was observed in recipients transplanted with these cells - probably reflecting the limitations of the *in vitro* conditions used. However, the correlation between leukemogenic potential of Lp30-mice and the ability of GMPs from these mice to immortalize suggests that this population might constitute the target for secondary mutations.

0634

PHARMACOLOGICAL TARGETING OF THE PI3K-AKT/PKB-MTOR PATHWAY ALTERS LOCAL ANGIOREGULATION IN ACUTE MYELOGENOUS LEUKEMIA

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Background. Acute myeloid leukemia (AML) is a heterogeneous malignancy characterized by clonal proliferation of immature myeloid cells in the bone marrow. Even though the AML cell biology has been extensively characterized, this has not translated into any major improvements in overall disease-free survival. New therapeutic agents that are more efficient and better tolerated than today's treatment are therefore needed. Bone marrow angiogenesis is involved in leukemogenesis and affect AML cells' chemosensitivity, and its regulation is mediated by the balance between pro- and antiangiogenic mediators that are constitutively released by either AML or stromal bone marrow cells. One possible approach to target angioregulation could be to modulate the constitutive AML cell release of regulatory cytokines through inhibition of intracellular signaling pathways. Among the emerging therapeutic alternatives in AML are agents acting on intracellular signalling pathways involved in regulation of cellular proliferation and viability. Two of these pathways involve phosphatidylinositol 3-kinase (PI3K) and mammalian target of rapamycin (mTOR), and these pathways are connected via the serine/threonine kinase Akt/Protein kinase B (PKB). The Akt/PKB is a client protein of heat shock protein 90 (HSP90) and modulates several substrates important for leukemogenesis, including the transcription factor nuclear factor-κB (NF-κB). *Aims.* To characterize effects of pharmacological targeting of the intracellular PI3K-Akt/PKB-mTor pathway on cell viability, apoptosis, proliferation and the constitutive release of pro- and antiangiogenic factors by primary human AML cells. *Methods.* Six pharmacological intervention were tested; (i) non-specific mTOR inhibitor rapamycin (1μM), (ii) specific inhibitor of mTORC1 temsirolimus (0,1μM), (iii) class I PI3K inhibitor GDC-0941 (1μM), (iv) class III PI3K and autophagy inhibitor 3-methyladenine (3-MA) (1mM), (v) HSP90 inhibitor 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) (1μM) and (vi) proteasome and NF-kB inhibitor bortezomib (0,1μM). These concentrations had a maximal effect on AML cell proliferation. Primary human AML cells from 60 unselected patients were cultured under highly standardized *in vitro* conditions before evaluation of proliferation (3H-thymidine incorporation), cell viability (flowcytomertic analyses with AnnexinV-propidiumiodide staining) and cytokine release (ELISA). *Results.* AML cells derived from various patients differed in their release of angioregulatory mediators and the response to pharmacological agents. The various inhibitors of the PI3K/Akt/mTOR pathway showed antiproliferative effects for a large subset of patients, but an unexpected growth enhancement was seen for a subset of patients, especially when testing temsirolimus and 3-MA. Rapamycin, temsirolimus and 3-MA had only minor effects on cell viability, while GDC-0941, 17-DMAG and bortezomib decreased the viability. All drugs inhibited the release of at least four angioregulatory cytokines (Table 1), and these effects could not be explained by altered AML cell viability. The inhibition was strong especially for Ang-1 and HGF that were significantly decreased by all agents. The mTOR inhibitors rapamycin and temsirolimus often showed weaker effects than the other drugs. Summary/*Conclusions.* Pharmacological agents that affect the PI3K-Akt-mTOR pathway alter proliferation and constitutive release of angioregulatory cytokines by primary human AML cells. Such effects may inhibit bone marrow angiogenesis and hence increase AML cells susceptibility to conventional chemotherapy.

Table 1. The effect of pharmacological agents on proliferation and cytokine release by AML cells (p<0.05 indicated by↓ p<0.001 indicated by↓↓)

	Number of patients with detectable proliferation or cytokine release (median level in controls)	Rapamycin	Temsirolimus	GDC-0941	3-MA	17-DMAG	Bortezomib
Proliferation	46 (6 791 cpm)	↓	-	↓	-	↓↓	↓↓
CXCL8	55 (11 981 pg/ml)	-	-	↓	-	↓↓	↓↓
CXCL9	31 (26.4 pg/ml)	-	↓	↓	↓	↓↓	↓↓
CXCL10	46 (190.6 pg/ml)	↓	↓	↓	↓↓	↓↓	↓↓
CXCL11	16 (246.8 pg/ml)	↓	-	↓	↓	↓	-
Ang-1	45 (120.9 pg/ml)	↓	↓	↓↓	↓↓	↓↓	↓↓
Ang-2	25 (19.0 pg/ml)	-	-	↓	↓	↓	↓
VEGF	17 (41.7 pg/ml)	-	↓	↓	-	↓	-
HGF	32 (396.5 pg/ml)	↓	↓	↓↓	↓↓	↓↓	↓↓

0635

MYELOPEROXIDASE EXPRESSION AS A POTENTIAL DETERMINANT OF PARTHENOLIDE-INDUCED APOPTOSIS IN LEUKEMIA BULK AND LEUKEMIA STEM CELLS

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Background. Since parthenolide (PTL) is an effective anti-leukemic agent, identifying molecular markers predicting response to PTL is important. Myeloperoxidase (MPO) may be an important determinant of leukemia cell sensitivity to oxidative stress-mediated apoptosis. *Aim.* To evaluate whether the PTL-induced apoptosis is critically modulated by MPO molecule in leukemia cells. *Methods.* We evaluated the role of MPO in determining the sensitivity of leukemia cells to PTL-induced apoptosis. *Results.* PTL-induced apoptosis via reactive oxygen species (ROS) generation acts to a greater extent in the MPO-high leukemia cell lines, as compared to MPO-low cell lines. Pretreatment of MPO-high

leukemia cells with MPO inhibitor, ABAH or ScAc, abrogated PTL-induced apoptosis, indicating that MPO molecule is critically involved in PTL-induced apoptosis. PTL-induced apoptosis was accompanied by downregulation of NF-κB, Bcl-xL, Mcl-1, XIAP, and survivin, and selectively observed in primary acute myeloid leukemia (AML) cells expressing higher level of MPO (≥ 50%), while sparing both AML cells with lower MPO and normal hematopoietic stem cells (HSC). PTL-induced apoptosis rate in the $CD34^+CD38^-$ cell fraction was greater in MPO-high AML cells, as compared to MPO-low AML ($P<0.01$) and normal CD34+ HSC ($P<0.01$) cells. NOD/SCID xenograft leukemia model revealed that PTL preferentially targets the AML $CD34^+CD38^-$ population. Summary/*Conclusions.* Our data suggest that MPO plays a crucial role in determining the susceptibility of leukemia cells to PTL-induced apoptosis. PTL can be considered a promising LSC-targeted therapy for AML with high level of MPO.

0636

MULTI-COLOR FLOW CYTOMETRIC MEASUREMENT FOR QUANTIFICATION AND IMMUNOPHENOTYPING OF LEUKEMIC STEM CELLS IN ACUTE LEUKEMIA

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Background. Leukemic stem cells are root of clonal growth in acute leukemia and responsible for growth and proliferation of leukemic blasts. They could remain as minimal residual disease after chemotherapy and cause relapse. Leukemic stem cells are defined as $CD34^+/CD38^-$ populations among leukemic blasts and often express CD123, CD44, CD96, CLL-1 and CD184 (CXCR4) which are rarely expressed on normal hematopoietic stem cells, and being studied as the targets of anti-leukemic therapy. *Aims.* We measured the proportions of leukemic stem cells and identified immunophenotypes of leukemic stem cells with clinical and laboratory findings of acute leukemia. *Methods.* Bone marrow aspirate of 75 patients who were newly diagnosed or relapsed as acute leukemia were measured for detection of leukemic stem cells from March to September 2009. At first, $CD45^+$dim or negative and low sideward scatter area in which leukemic blasts exist was gated on a FACSCanto flow cytometer (BectonDickinson, San Jose, CA, USA), and then proportion of $CD34^+/CD38^-$ leukemic stem cells among leukemic blasts were measured. After then proportions of $CD123^+$, $CD44^+$ and $CD184^+$ leukemic stem cells were obtained. The proportions and immunophenotypes of leukemic stem cells were compared with patients' WBC count, bone marrow blast proportion, morphologic features of blasts and molecular genetic or cytogenetic findings and clinical courses which were reviewed from electronic medical record. *Results.* Median leukemic stem cell proportion among leukemic blasts was 3.1% (0.0-63.4%) in all 75 patients with acute leukemia (54 acute myeloid leukemia (AML), 17 acute B-lineage lymphoblastic leukemia (B-ALL) and 4 acute T-lineage lymphoblastic leukemia (T-ALL)) at the time of diagnosis. B-ALL patients showed higher leukemic stem cell proportions (median 20.2%, 0.0-63.4%) than AML (median 2.5%, 0.0-54.7%) and T-ALL patients (median 0.2%, 0.0-0.8%) ($P=0.008$). CD123 was tend to be highly expressed on leukemic stem cells in AML and B-ALL ($P=0.050$), CD44 was highly expressed on leukemic stem cells in AML and T-ALL ($P<0.001$). There was a negative correlation between leukemic stem cell proportion and WBC count ($P=0.039$). But proportions of leukemic stem cells were not correlated with proportions of bone marrow blasts ($P=0.586$). There were no differences in proportions and immunophentypes of leukemic stem cells according to the molecular genetic or cytogenetic findings. Comparing complete remission (CR) group and non-CR group after chemotherapy, proportions of leukemic stem cells were significantly low in CR group with AML. Otherwise, there were no significant relationships between characteristics of leukemic stem cells and relapse rate. Summary/*Conclusions.* We were able to measure the proportions of leukemic stem cells among leukemic blasts fast and simply by using multi-color flow cytometry. The proportions of leukemic stem cells are different according to the diagnosis of acute leukemia, CD123 and CD44, being studied as the targets of anti-leukemic therapy are also differently expressed among leukemic stem cells. The lower proportions of leukemic stem cells in AML patients with complete remission state indicated that measurement of the proportion of leukemic stem cells might be helpful to predict the prognosis of AML.

0637

GEFITINIB ENHANCES ARSENIC TRIOXIDE (AS2O3)-INDUCED DIFFERENTIATION OF ACUTE PROMYELOCYTIC LEUKEMIA CELL LINE

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Gefitinib (Iressa, ZD1839), a selective epidermal growth factor receptor tyrosine kinase inhibitor, inhibits growth, invasion and colony formation of various cancer cells. However, little is known about the effect of combination of gefitinib and arsenic trioxide (ATO) on differentiation of acute promyelocytic leukemia (APL). Therefore, we investigated whether gefitinib had any role in the ATO-induced differentiation of NB4 cells (APL cell line). As shown in Figure 1, gefitinib (10 μM) significantly enhanced ATO-induced differentiation of NB4 cells.

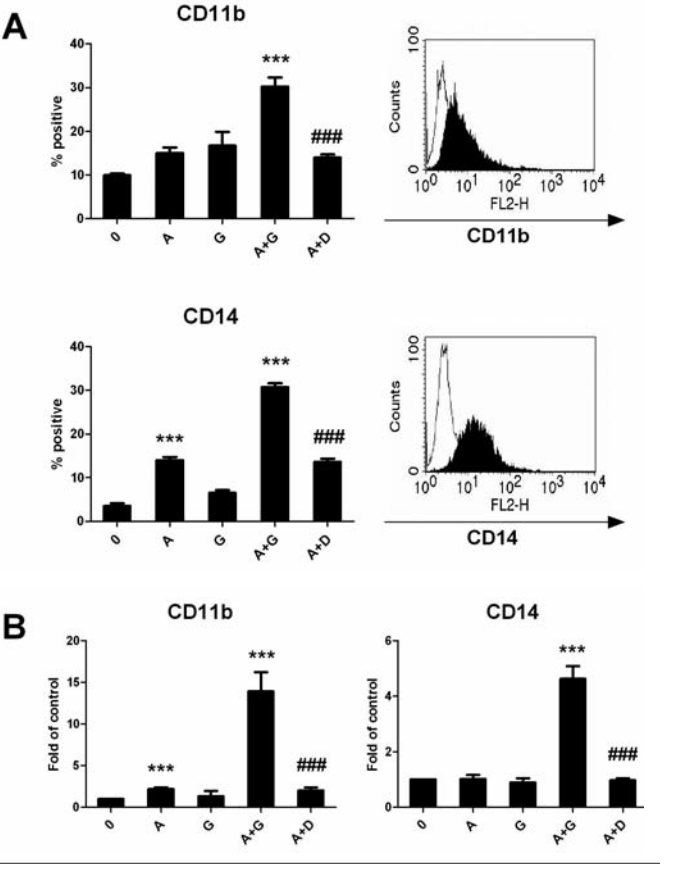

Figure 1. Synergy effects of gefitinib and ATO.

Induction of differentiation was assessed by measuring the expression of myeloid marker proteins including CD11b and CD14 using flow cytometric analysis. Treatment of NB4 cells with arsenic trioxide (0.6 μM) alone for 3 days resulted in 15% CD11b-positive cells and 14% CD14-positive cells, whereas gefitinib alone (10 μM) had no effect on the expression of differentiation markers such as CD11b and CD14 of NB4 cells. However, NB4 cells in ATO combined with gefitinib showed significantly enhanced expression of CD11b and CD14 by 3 and 8.8-fold, respectively (30.3% CD11b-positive cells and 30.8% CD14-positive cells). Moreover, we also measured the mRNA levels of CD11b and CD14 using QRT-PCR. The results revealed that expression of CD11b and CD14 increased 13.9-fold and 4.6-fold in response to augmentation of gefitinib in ATO-treated NB4 cells. The ATO (0.6 μM) with DMSO was used as a negative control to compare with the combination of ATO and gefitinib. The expression of differentiation markers and QRT-PCR of the arsenic trioxide with DMSO were similar to those of ATO alone. These results indicated that gefitinib played an important role on the enhancement of ATO-induced differentiation of NB4 cells. Moreover, the ERK pathway was necessary for the enhancement of gefitinib in ATO-induced differentiation, measured by CD11b and CD14 expression on NB4 cells. Therefore, our data indicated that

gefitinib can play a potential role as an adjunctive differentiation agent in APL.Mitogen-activated protein kinase (MAPK) and ERK were reported to play an important role on AML differentiation.[14] We assessed whether ERK or p38 MAPK pathway was related to gefitinib-enhanced differentiation of NB4 cells treated with ATO. We pretreated NB4 cells with 20 and 15 μM of PD98059 (an ERK inhibitor) and SB203580 (a p38 MAPK inhibitor) for 1 hr, and then stimulated the cells with 10 μM gefitinib in ATO-treated NB4 cells. Cells were collected and myeloid cell surface marker CD11b and CD14 expression were measured using flow cytometric analysis. CD11b and CD14 expression, or macrophage-like cells differentiation increased after exposure to gefitinib in ATO-treated NB4 cells. However, PD98059 treatment led to significant reduction of CD11b and CD14 expression in gefitinib-enhanced NB4 cells. Also, SB203580 pretreatment alone showed no significant effect on the CD11b expression when compared with a control group, whereas it significantly suppressed CD14 expression in gefitinib and ATO-treated NB4 cells. Therefore, the results indicated that the ERK pathway is essentially required for gefitinib enhancement of ATO-induced differentiation of NB4 cells. And p38 MAPK pathway is partially related to the gefitinib/ATO-induced differentiation of NB4 cells.

0638

DOWN-REGULATION OF NR4A3 IS ASSOCIATED WITH DIFFERENTIATION IMPAIRMENT DURING MYELOID LEUKEMOGENESIS

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Background. Acute myeloid leukemia (AML) is a relentless hematological malignancy characterized by overproduction of hematopoietic precursor cells with impaired differentiation. Increasing evidences suggest that the development of AML is a multistep process that requires collaboration of at least two classes of genetic aberrations: class I aberrations confer a proliferation advantage to hematopoietic cells, whereas the class II aberrations serve primarily to impair hematopoietic differentiation. Orphan receptors Nr4A1 and Nr4A3 have been implicated to be homeostatic regulators of proliferation, apoptosis and differentiation. Recently it was reported that abrogation of these two proteins in mice led to development of AML. Besides, down-regulation of both genes was a common feature in leukemic blasts from AML patients, irrespective of karyotypes. These findings suggest that silencing of both genes plays a critical role in myeloid leukemogenesis. *Aims.* Nr4A1 has been proposed to contribute to cell apoptosis; suppression of its expression is supposed to be correlated with survival advantages (class I aberration). Nonetheless the function of Nr4A3 in myeloid leukemogenesis remains unclear. We hypothesize that suppression of Nr4A3 may impair the hematopoietic differentiation (class II aberration). *Methods.* K562 cells were treated with 12-O-Tetradecanoylphorbol-13-acetate(TPA) to induce megakaryocytic differentiation. NB4 cells were treated with all-trans-retinoic acid(ATRA) to induce granulocytic differentiation. The Nr4A3 expression levels were determined before and after differentiation induction in these two cell lines. The effect of Nr4A3 on leukemic cells differentiation was also investigated by Nr4A3 knock-down using siRNA. For clinical validation, the Nr4A3 expression in archived bone marrow (BM) samples from a cohort of chronic myelocytic leukemia (CML) patients in various disease phases and leukemia cells from peripheral blood of a patient with fresh acute promyelocytic leukemia (APL) treated with ATRA was analyzed by quantitative real-time PCR (Q-PCR). The expression of megakaryocytic differentiation marker PDGF was determined by Q-PCR, and that of granulocytic differentiation marker CD11b was determined by flowcytometry. Transfection of Nr4A3 siRNA was done by electroporation. Results. Differentiation of K562 and NB4 cells were successfully induced by TPA and ATRA, respectively, as shown by morphological appearance and PGDF and CD11b expression. The induction of differentiation also led to obviously increased Nr4A3 expression in both cell lines. Transfection of siRNA into K562 cells not only knocked down Nr4A3 expression after TPA treatment but also suppressed K562 differentiation demonstrated by decreased PDGF expression. These data suggested that Nr4A3 expression is necessary for leukemic cell differentiation *in vitro*. In CML patients' bone marrow cells, Nr4A3 expression did not decrease when the disease was in chronic phase, but it was significantly decreased when the disease progressed to acute blastic phase. In peripheral blood cells of an APL patient, Nr4A3 expression was low before ATRA treatment but was elevated after 2 days of ATRA treatment. These clinical findings are compatible with the hypothesis that Nr4A3 expression levels are associated with leukemic cell differentiation status. *Summary/Conclusions.* Down-regulation of Nr4A3 is associated with differentiation impairment during myeloid leukemogenesis. Induction of Nr4A3 expression could be a target of differentiating therapy for AML.

0639

IN VITRO AND *IN VIVO* EFFECT OF AN ORAL IRON CHELATOR DEFERASIROX IN MYELOID LEUKEMIA CELLS

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Background. Iron is an essential component for cell proliferation and viability, and it has been reported that iron depletion by chelator inhibits the proliferation of cancer cells, including hepatoma, neuroblastoma and leukemia. Among the different kind of iron chelators, deferoxamine is the most widely studied molecule as an antitumor agent. Deferasirox (DFS) is a new oral iron chelator and there are few reports regarding *in vitro* and *in vivo* effect on myeloid leukemia cells. *Aims.* This study was undertaken to evaluate the *in vitro* and *in vivo* effect of DFS in myeloid leukemia cells and to define the involved molecular mechanism. *Methods.* The effect of deferasirox on two human leukemic cell lines (HL-60 and KG-1) was determined by cell proliferation (MTT assay), cell cycle analysis, and apoptosis assay. MTT assay was done with various concentrations (5, 10, and 50 μM) of DFS for 24, 48, or 72 h. Apoptosis was evaluated by flow cytometry with use of Annexin V and propidium iodide (PI) and by caspase activation assay. Western blotting was used to investigate the involved signal(s) in apoptotic pathways. Tumor xenografts in nude mice (n=14 for each cell line) were used to evaluate the *in vivo* effect of DFS. All *in vitro* experiments were repeated at least three times. *Results.* MTT assay showed that DFS had antiproliferative effect on both myeloid leukemia cell lines and this cytotoxicity was a dose- and time-dependent manner. Cell cycle analysis revealed that subG1 fraction was increased dose-dependently. To determine whether the cytotoxicity induced by DFS was due to apoptosis in HL-60 and KG-1, we measured the fraction of early apoptosis [Annexin V(+)/PI(-)] and caspase-3 and -9 activities. The percentage of Annexin V(+)/PI(-) cells was increased significantly and caspase-3 and -9 activities were also increased by colorimetric assay. Western blot analysis revealed that expression of cleaved PARP, caspase-3, and -9 was increased, indicating involvement of caspase-3/-9 pathway in DFS-induced apoptosis. For *in vivo* experiments, we injected 5×10^6 (HL-60) and 1×10^7 (KG-1) cells into nude mice, respectively. Treatment group (DFS 20 mg/kg) showed statistically significant decrease in tumor mass compared with control group. Summary/*Conclusions.* This study demonstrated that a new oral iron-chelating agent DFS had apoptotic effect on myeloid leukemia cells, and apoptosis was dependent on caspase-3/caspase-9 pathway. DFS might be beneficial for iron-overloaded patients with myeloid leukemia, not only from iron chelation but also from reduction of leukemia cells.

0640

IMPACT OF MOLECULAR MARKERS ON THE OUTCOME OF ACUTE MYELOID LEUKEMIA WITH INTERMEDIATE-RISK CYTOGENETICS

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Chromosomic alterations help to classify Acute Myeloid Leukemia (AML) in prognostic groups. The intermediate group (IG) described by the Medical Research Council (MRC), with overall survival (OS) of 35-40% at 5 years, is heterogeneous and includes patients with normal karyotype (NK) (50%) and others with cytogenetic abnormalities not associated to favorable or bad prognosis. Several studies have shown that the prognosis in the IG is more accurately defined by molecular characterization including mutations in NPM1, FLT3-ITD and CEBPA, as well as expression of new genes such as the BAALC, EVI1, MN1 and ERG. Objective. To analyze the impact on outcome clinical and biological features, including the above mentioned molecular markers, in AML patients belonging to the IG who achieved a complete remission (CR). *Patients and Methods.* 197 patients with available samples for gene studies and treated under the CETLAM AML-03 protocol were included in the study. Parameters analyzed were complete CR rate, and disease free survival (DFS), Kaplan Meier analysis of time-dependent variables, and

Cox regression for multivariable studies. *Results.* The median age of the series was 53 years (range 20-73); 8% of the patients died during induction chemotherapy, 81% achieved complete remission, and 11% showed refractoriness to treatment. In the multivariate analysis, the presence of NPM1 or CEBPA mutations without FLT3-ITD was associated to increased CR rate (95% *vs.* 75%, P= 0.001). OS of the cohort at 4 years was 41±4,6%, DFS 44±4.6% and relapse probability (REL) 52±5%. Adverse prognostic factors for the OS in the multivariate analysis were age over 50 years (>50y *vs.* <=50y P<0.001, odd ratio [OR]: 2.5 CI95%: 1.6-3.9), and the absence of a favorable genotype (NPM1+,or CEBPA+/FLT3-ITD-)(P<0.001,OR:4, CI95%:2.2-7.5). Regarding DFS, the adverse prognostic factors were age over 50 years (p:0.023, OR: 1.7, CI95%:1.1-2.8), a white blood cell count (WBC) over 100×10^9/L (>=100 *vs.* <100, P:0.050, OR:1.7, CI95%:1-3) and the absence of a favorable genotype (P:0.003, OR:2.3, CI95%1.3-4). For REL, the independent adverse factors were, older age (>50y) (P:0.036, OR:1.7, CI95%:1-2.9), the absence of a favorable genotype (P:0.003, OR:2.6, CI95%:1.3-4.8), and the over-expression of BAALC gene (P:0.011, OR:2.6 CI95%:1.2-5.1). *Conclusions.* In AML with intermediate cytogenetic risk, the over-expression of BAALC was an adverse prognostic factor for relapse rate. Regarding DFS and OS, the well established prognostic factors such as age, leukocyte count and NPM1, CEBPA and FLT3 status were confirmed in this series. The results presented here, confirmed the need to adjust therapeutic decisions based in clinical and biological features.

0641

ANTI-LEUKEMIC EFFECT OF SODIUM METAARSENITE (KML001) IN ACUTE MYLOGENOUS LEUKEMIA WITH BREAKING-DOWN THE RESISTANCE OF CYTOSINE ARABINOSIDE

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Background. Arsenic compounds have been used in traditional medicine for several centuries. Especially, arsenic trioxide (As_2O_3) has proven effective in the treatment of acute promyelocytic leukemia (AML M3 subtype). A drawback of arsenic trioxide is that it is administered intravenously, and not effective in the treatment of acute mylogenous leukemia (AML) except AML M3 subtype. Sodium metaarsenite ($NaAs_2O_3$: code name KML001) is an orally bioavailable arsenic compound with potential anti-cancer activity. However, the effect of KML001 has not been well studied in AML. *Aims.* Firstly, to determine the anti-leukemic effect of KML001 in AML, and to compare the efficacy with arsenic trioxide, secondarily, to investigate the mechanism of anti-leukemic effect of KML001. *Methods.* Eleven AML cell lines were used in this study including Ara-C (cytosine arabinoside) resistant HL-60 (HL-60R) cells. AML blasts were isolated from 4 AML patients (2 M1 and 2 M2 subtypes) after obtaining informed consents. Cellular inhibition was measured by MTT assay. Expression of molecules was done by western blot. Analysis of cell cycle was used by flow cytometry. Transcriptional expression of catalytic subunit of telomerase, hTERT, was done by real-time PCR. *Results.* KML001 inhibited the cellular proliferation in all AML cell lines and primary AML blasts as well as HL-60R cells in a dose-dependent manner with IC_{50} of 5×10^{-8}M. While KML001 effectively inhibited cellular proliferation of HL-60 cells (IC_{50}; 5×10^{-8}M) as well as HL-60R cells (IC_{50}; 1×10^{-8}M), and its anti-leukemic effect was almost same as Ara-C (IC_{50}; 5×10^{-8}M), Ara-C did not inhibit cellular proliferation in HL-60R as expected. Furthermore, arsenic trioxide was not effective in primary AML blasts and AML cell lines including HL-60 R cells. KML001 (1×10^{-7}M) was induced G1 cell cycle arrest which was associated with decreased expression of cyclin D1, cyclin E1, CDK1 (cdc2p34), CDK4, and CDK6. While KML001 increased the p21 and p27 levels, and enhanced their bindings with CDK4 in HL-60 cells, the expression of p21 and p27 bound CDK2 was observed in HL-60R cells. Apoptotic molecules of Bcl-2, proform of caspase-3 and caspase-9 were decreased, in contrast, expression of PARP was increased in HL-60 and HL-60R cells treated with KML001. Real-time PCR with RNA extracted from KML001-treated HL-60 and HL-60R cells showed a significant reduction of catalytic subunit of telomerase, hTERT, at 12 hr. When treated KML001, DNA damage molecule (γ-H2AX) in HL-60 and HL-60R cells was increased. In addition, KML001 inhibited the activation of STAT1, 3, 5, NF-κB (p65 and p50 subunits), pAKT and PI3K in a time-dependent manner. On the other hand, activated PTEN was up-regulated.Summary/*Conclusions.* KML001, sodium metaarsenite, demonstrated anti-leukemic effect via various mechanisms including cell cycle arrest, induction of apoptosis, and inhibition of JAK/STAT and PI3K pathways. Especially, KML001 might target telomerase with DNA damage. Furthermore, it is probable that KML001 may overcome the resistance of chemotherapeutic agents. Collectively, KML001 may be a candidate agent for the treatment of *de novo*, refractory and relapsed AML.

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FLOW-CYTOMETRIC MULTIPLEX IMMUNOBEAD ASSAY FOR THE DETECTION OF CBFβ-MYH11 AND AML1-ETO FUSION PROTEINS IN ACUTE MYELOID LEUKEMIA

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The prognostic classification of acute myeloid leukemias (AML) is generally based on the presence or absence of specific genetic aberrations-particularly fusion genes-that are detected by karyotyping, FISH or PCR. These techniques are laborious and demand specialized laboratories. Therefore fast and easy research-based cytometric bead array (CBA) assays (BD Biosciences) are being developed for detection of fusion proteins in cell lysates of leukemic cells that contain these well-defined fusion gene aberrations. Here we present data of a multiplex AML "core-factor"CBA which detects the AML1-ETO and CBFβ-MYH11 "core-factor"fusion proteins. AML patients harboring either of those fusion proteins comprise a biologically distinct and prognostically favorable subgroup of patients who often achieve long-lasting complete remissions. Therefore accurate identification of CBFβ-MYH11/ inv(16) and AML1-ETO/ t(8;21) positive cases is essential. Monoclonal antibodies were generated against the fusion proteins (both N- and C-terminus). Based on the recognized epitopes, the antibodies should recognize all variants of the CBFβ-MYH11 fusion proteins which result from translocations in the different breakpoint cluster regions. In the CBA, catcher antibodies were coupled to separate CBA beads and the detector antibodies with fluorochrome-labeling. A specific signal in the flow cytometer is only detectable when the relevant fusion protein is present in the analyzed sample. Since wild-type proteins are not recognized by the antibody couples, no signal should be obtained when samples are analyzed that do not contain the fusion protein. To limit the number of cells required for analysis, the two assays were combined into a multiplex so-called "AML core-factor"CBA. The multiplex assay appeared specific (cell lines that express other fusion proteins were not detected) and sensitive since it detects at least 10-15 % of fusion protein positive leukemic cells diluted in normal PB-MNCs or WBC's. The suitability of the multiplex AML core-factor CBA was tested in five EuroFlow laboratories: 63 myeloid and monocytic AML samples were analysed (blood and bone marrow).The presence or absence of CBFB-MYH11 or AML1-ETO transcripts in the specimens was determined by RQ-PCR analysis according to the EAC program. The performance of the CBA was assessed by parallel analysis of well-defined positive and negative control samples. The multiplex AML assay had full concordance with the RQ-PCR results (18/18 positive and 45/45 negative) with identification of all AML1-ETO (5/5) and CBFβ-MYH11 (13/13) positive patients. The multiplex AML core-factor CBA is suitable for accurate and simultaneous detection of CBFβ-MYH11 and AML1-ETO containing samples. The assay is specific and can be performed within 4-5 hours in a routine diagnostic setting, without the need of special equipment other than a flow cytometer. The novel CBA will enable fast and easy classification of AML patients that express CBFβ-MYH11 and AML1-ETO fusion proteins. These patients can be included at an early stage in the right treatment protocols, much faster than by use of current molecular techniques. The CBA can be run in parallel to routine immunophenotyping and is particularly attractive for clinical settings without direct access to molecular diagnostics.

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GENE EXPRESSION ANALYSIS IN AML CELL LINE MV4-11 FOLLOWING TREATMENT WITH THE ANTI-CANCER APTAMER AS1411

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Background. AS1411 is a 26-base DNA aptamer which is shortly due to enter a phase IIb clinical trial in acute myeloid leukaemia (AML). In

an initial phase II trial in AML, combination of AS1411 with cytarabine produced an increased response rate compared to cytarabine alone, and AS1411 was well tolerated. When cells in culture are exposed to AS1411, a cytostatic effect is observed initially, followed by cell death after 72 to 96 hours. Several mechanisms have been described by which AS1411 exerts these effects; we performed a gene expression microarray analysis to evaluate which cellular pathways and functions were modulated by AS1411 treatment. We also sought to identify biomarkers for drug activity which could be translated to use in the clinic in the forthcoming phase IIb trial. *Results.* The AML cell line MV4-11 was exposed to AS1411 at two concentrations; these approximated concentrations observed in patient plasma following two different doses of AS1411 in the prior phase II trial. mRNA was isolated from cells at 24 and 72 hours, time points associated with cytostatic and cytotoxic effects, respectively. Controls at time zero and in the absence of drug were also analysed. The Affymetrix plus 2™ arrays were processed and the raw data were normalised according to the Rosetta Resolver model. The gene lists for each time point (4 replicates) were then clustered non-hierarchically and distributed into pathways using MetaCore™. Gene clusters were separately generated to identify specific genes which were up- or down-regulated at different doses and different time points. These were then compared to identify those that were regulated by a common pathway. Pathways and regulatory networks that were either up- or down-regulated after exposure to AS1411 included nuclear import/export, nucleotide biosynthesis/metabolism, DNA damage response and pathways related to cell cycle control and cell death (including genes in the p53 signalling pathway). While some of these activities had been shown previously to be modified upon AS1411 exposure, other findings are novel. A number of genes showed marked changes in expression following AS1411 exposure; these are undergoing validation by quantitative PCR as potential biomarkers. They include upregulated genes TP53i3 and p21 (p53 signalling pathway), RDM1 (DNA damage), YPEL3 and DAPK1 (proliferation and apoptosis) as well as individual genes such as GNG7. Several genes, including INO80C, exhibited a marked dose dependent down regulation. *Conclusions.* This work demonstrates that a number of cellular activities are modulated by AS1411, and provides important information for the future clinical development of AS1411, including pointers to the selection of biomarkers for analysis of blood and bone marrow samples from patients in the forthcoming phase IIb study in AML.

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ANKYRIN REPEAT AND KH DOMAIN CONTAINING 1 (ANKHD1), A NEW SCAFFOLDING PROTEIN, REDUCES APOPTOSIS AND IS MODULATED IN LEUKEMIC CELLS

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Background. Ankyrin repeat and KH domain containing 1 (ANKHD1) is the human homolog of the Drosophila melanogaster Mask (Multiple ankyrin repeat and single KH domain protein), a protein involved in photoreceptor differentiation, cell survival and proliferation. ANKHD1 associates with SHP2 in neoplastic cells and all its isoforms are upregulated during hematopoietic cell differentiation. ANKHD1 knockdown of in Hela cell lines was associated with increasing of apoptosis levels and caspase activation However, the exact contribution of ANKHD1 in leukemogenesis is still unknown. *Aims.* The aim of this study was to analyze the gene expression profile in ANKHD1-depleted cells, compared to control cells. We also evaluated apoptosis levels in leukemia cell line submitted to ANKHD1 overxpression and ANKHD1 expression after treatment with immunomodulator drugs. *Methods.* Analysis of gene expression profile of ANKHD1-depleted cells was performed using a microarray approach (codelink,GE technologies) in siRNA-treated cells, analyzed by Ingenuity Pathway Analysis. Using real-time quantitative polymerase chain reaction (qRT-PCR), ANKHD1 expression was analysed in bone marrow mononuclear cells (BMNC) of patients with acute leukemia (AL) at the time of diagnosis or in BMNC from healthy donors. A total of 38 adult cases diagnosed with acute leukemia at the Hematology and Hemotherapy Center of the State University of Campinas were studied, including 7 ALL, 1 biphenotypic acute leukemia and 30 AML (1 M0, 5 M1, 6 M2, 3 M3, 10 M4, 3 M5, 1 M6, 1 M7) based on the French-American-British (FAB) classification and normal bone marrow donors (n=11).The hyperxpression was done by AMAXA®nucleofector® electroporation and apoptosis levels were measured in percent of annexin 5(Molecular probes) positive cells under and without the action of camptothecin apoptosis induction. Leukemia cell lines were treated with Interferon α or Thalidomide for 18 and 16 hours respectively and ANKHD1 expression was analyzed by qRT-PCR. Statistical analysis was performed by Mann-Whitney test. *Results.* BMF, a pro-apoptotic "BH3-only protein"of the BCL2 family, was upregulated in ANKHD1-depleted cells in microarray analysis. qRT showed a reduced expression of ANKHD1 transcripts and an upregulation of BMF in leukemia primary samples ($P<0.0001$ and $P<0.001$ respectively), corroborating our microarray *Results.* ANKHD1mRNA was upregulated in leukemia cell lines under Interferon α or Thalidomide treatment. Finally, ANKHD1 hyperexpression protects leukemic cell from apoptosis induction by camptothecin. *Conclusions.* The high levels of BMF expression is associated with bad prognosis in leukemia. Our results show that dowregulation of ANKHD1 is associated with high levels of BMF in primary leukemic samples and ANKHD1 overexpression induced decreasing in apoptosis. These results indicate that ANKHD1 is likely a novel scaffolding protein involved in apoptosis signaling and/or cellular proliferation processes and could be involved in leukemogenesis.

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IMPACT OF ADDITIONAL CHROMOSOMAL ABNORMALITIES (ACAS) IN PATIENTS WITH ACUTE PROMYELOCYTIC LEUKEMIA 10 YEARS' FOLLOW-UP RESULTS OF THE JAPAN ADULT LEUKEMIA STUDY GROUP APL97 STUDY

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Background. A combination of all-trans retinoic acid (ATRA) and chemotherapy (CT) has improved the clinical outcome of APL. However, considerable numbers of patients still relapse after an initial response. Although prognostic factors for APL have been studied, the prognostic impact and clinical characteristics of ACAs in APL remains controversial. The results differ among previous papers (De Botton *et al.*, Hernandez *et al.*, Schlenk *et al.*). Recently, Cervera *et al.* found in the largest study that ACAs were associated with lower relapse-free survival in univariate analysis. *Aims.* Large prospective studies with longer follow-up are needed to evaluate whether ACAs have an impact on the efficacy of treatment with ATRA and CT. We analyzed the prognostic importance of ACAs in 10 years' follow-up of a large prospective study. *Methods.* Among 302 patients with newly diagnosed APL enrolled in this study (Asou *et al.*, 2007), 225 patients were assessable. The patients had been continuously registered from May 1997 to June 2002. Informed consent was obtained before registration. Clinical outcomes were updated on January, 2009, and the median follow up period is 7.3 years. Clinical characteristics were analyzed in relation to ACAs. In addition, CR rate, relapse rate (RR), OS and disease-free survival (DFS) were assessed in APL patients with or without ACAs. *Results.* ACAs were observed in 67 patients (29.7%). Trisomy 8 was found in 21 cases (31%). Other ACAs were found involving chromosome 15 and 17 in twelve (18%), chromosome 9 in seven (11%), chromosome 7 in six (9%), chromosome 6 in four (6%). No clinical or biological differences were found between patients with or without ACAs, except for the frequency of M3v, which was significantly lower in those with ACAs (P=0.04). The CR rates in patients with or without ACAs were 97% and 95%, respectively (P=0.72). DFS and OS were not different between the two groups at 10 years (68% *vs.* 71%, P=0.59; 91% *vs.* 84%, P=0.18; respectively). The cumulative incidence of relapse was not different between the two groups (26% *vs.* 22%, P=0.51). The cumulative incidence of early death at 50 days in the patients with or without ACAs was 2% and 6%, which was not significantly different (P=0.16). Additionally, the clinical outcomes were analyzed among subgroups of patients with ACAs. DFS and OS were not significantly different between ACAs patients with or without trisomy 8 (70% *vs.* 67%, P=0.58; 95% *vs.* 89% , P=0.37; respectively). Other abnormalities including chromosome-7, -9, -15 and -17 did not influence outcomes. *Conclusions.* The prognostic impact of ACAs has not been elucidated in this study. One of the reasons in the experimental difficulty is the clinical outcome of APL has been considerably improved due to the introduction of ATRA. It might become more difficult to determine significant prognostic factors in the study of APL. However, we should continue to conclude it in larger prospective studies after the introduction of more specific molecular-targeted agents, such as arsenic trioxide and gemtuzumab ozogamicin.

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C-KIT MUTATION IS A SIGNIFICANT PROGNOSTIC FACTOR OF T(8;21) AML: IMPORTANCE OF HIGHLY SENSITIVE Q-PROBE METHOD

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Background and *Aims.* t(8;21) AML is thought to have a better prognosis than other AMLs. Nevertheless, only approximately 50% of patients were alive at 5 years. This suggests that some patients have more aggressive leukemic phenotypes and indicates the need for predictive marker of relapse. Recently, several groups reported that c-kit mutation defined an unfavorable subgroup in t(8;21) AMLs. However the rate of c-kit mutation has been reported to be approximately 20% at diagnosis of core binding factor leukemias. In the present study, we analyzed samples collected at diagnosis to investigate the role of c-kit mutations and FLT3-ITD in t(8;21) AML. *Methods.* We analyzed c-kit mutation and FLT3-ITD among t(8;21) AML patients diagnosed between 1991-2009 at the Nippon Medical School Hospital. Exon 8 and 17 of c-kit were analyzed by direct sequencing method and Q-Probe system (ARKRAY Inc. Kyoto, Japan). Q-Probe method was a highly sensitive mutation screening system using mutation specific guanine quenching probe (Leukemia Res 32;1462-1467,2008). FLT3-ITD was analyzed by PCR amplification. *Results.* 26 patients were enrolled. All of them successfully achieved CR, but 15 patients (57.7%) relapsed. Using direct sequencing method, c-kit mutations were found in 5 patients out of 26 patients (D816V: 3 patients, N822K: 2 patients). Next we analyzed to detect small amount of mutations by Q-Probe method and D816V in 1 patient and N822K in 6 patients were newly found at diagnosis in the patients negative by direct sequencing method. Totally, c-kit mutations were found in 12 patients out of 26 patients at diagnosis (46.2%, D816V: 4 patients, N822K: 8 patients) using Q-Probe method. To evaluate the importance of c-kit mutations as a predictive factor of relapse, we analyzed the cumulative incidence of relapse (CIR) of these patients using Kaplan-Meier method. Relapse rate of all cases was 57.7% and median time to relapse was 16 months. In direct sequencing method, the CIR at 2 years tended to be higher for patients with c-kit mutations, but there was no significant difference (P=0.3970, wild type 54.9% *vs.* c-kit mutation 80.0%). On the other hand, when we analyzed using Q-Probe method, the CIR at 2 years was significantly higher for patients with c-kit mutations with the p value of 0.0193 (wild type 41.2% *vs.* c-kit mutation 81.8%). We detected FLT3-ITD in 2 patients at diagnosis, then we analyzed the CIR accordingly to the presence of c-kit mutation or FLT3-ITD. The CIR at 2 years was much higher for patients with c-kit mutation (Q-Probe method)/FLT3-ITD than the patients without mutations (P=0.0069, wild type 35.5% *vs.* c-kit/FLT3-ITD 83.3%). As for the position of c-kit mutations, the function of D816V was thought to be closely related to the relapse of t(8;21) AML. *Conclusions.* In this study, we showed that c-kit mutation was a significant poor prognostic factor for t(8;21) AML. And we also revealed the importance of using highly sensitive Q-Probe method. It will be necessary to consider therapeutic strategy including up-front stem cell transplantation and/or tyrosine kinase inhibitors for t(8;21) AML patients with c-kit/FLT3-ITD mutations.

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PREDICTIVE VALUE OF AN IN VITRO BIOLUMINESCENT ASSAY FOR RAPID ASSESSMENT OF RESPONSE TO CYTARABINE AND FLUDARABINE IN PATIENTS WITH ACUTE LEUKAEMIA

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Cytarabine (Ara-C) is the mainstay of treatment for acute non lymphoblastic leukaemia (ANLL). Currently there is no rapid, inexpensive test to assess patient sensitivity to Ara-C or combination chemotherapy prior to treatment. Over 30% of patients with ANLL fail to respond to Ara-C with potential causes of resistance including decreased hENT-

1 transporter activity and over expression of cytidine deaminase (cdd). We have previously reported a bioluminescent 8-hour assay which assesses Ara-CTP levels in leukaemic cell lines and patient samples independently of the cause of patient resistance (Anderson *et al.,* Blood 2009; 114(22): p643). In theory any agent capable of potentiating generation of Ara-CTP from Ara-C can also be tested on the biosensor system. Here we present results using the 8-hour assay for combination therapy screening, as tested on seven ANLL cell lines and an initial cohort of seven patients with ANLL, dosed with Ara-C alone or in conjunction with the purine analogue fludarabine and granulocyte-colony stimulating factor (G-CSF). The 8-hour assay was optimised for combination therapy using a range of pre-incubation periods based on the protocol for *in vitro* dosing proposed by Ahlman *et al*. (Leukaemia 2001; 15(1): 69-73). The optimal dosing schedule required 4 hours pre-incubation with fludarabine (5 μM) followed by the 8-hour protocol previously described (Anderson *et al.,* Blood 2009; 114(22): p643). Where appropriate G-CSF (5 ng/mL) was dosed 24 hours prior to treatment with fludarabine and/or Ara-C (FLA). The Ara-C concentration used *in vitro* was 25 μM which is equivalent to 2 g/m^2 in the clinical setting. ANLL samples were sourced at presentation from patients with blast burdens of >80% in bone marrow. The *in vitro* FLA dosing schedule was initially tested on seven ANLL cell lines with known response to Ara-C (CCRF-CEM, K562, MV4-11, HEL, KG-1a, HL-60 and THP-1) and these results were correlated with a commercially available 3-day cytotoxicity assay (CellTiterGlo™, Promega). A significant response to FLA dosing was observed for K562, THP-1 and MV4-11 cell lines, which are highly resistant to Ara-C using the 8-hour assay. These results were corroborated by the 3-day cytotoxicity assay. For the patient cohort the distribution of FAB sub-types was M4 (30%), M2 (14%), secondary AML (14%), M0 (14%), biphenotypic AML (14%) and Ph+ ALL (14%). Patient ages ranged from 27 to 71 (median 53 years) and included five peripheral blood and three bone marrow samples. In four samples the 8-hour assay showed a significant improvement in blast cell sensitivity to combination treatment compared with Ara-C alone ($P<0.05$ in all cases). Confirmation of the predictive capacity of the 8-hour assay was observed in two of these patients: one was initially resistant to daunorubicin/Ara-C, and achieved remission with FLAG-Idarubicin; the second also successfully achieved remission with FLAG-Idarubicin. This bioluminescent assay system offers a valuable tool in predicting response in patients receiving Ara-C or fludarabine/Ara-C. This may be of importance in patients demonstrating chemoresistance, at presentation and at relapse.

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SAFETY AND EFFICACY OF ALL-TRANS RETINOIC ACID (ATRA) AND ARSENIC TRIOXIDE (ATO) COMBINATION THERAPY IN ACUTE PROMYELOCYTIC LEUKEMIA

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Introduction. Acute Promyelocytic Leukemia (APL) is considered as one of the curable malignancy. The standard therapy consists of all-trans Retinoic Acid (ATRA) along with an anthracycline with or without cytarabine. The relapse/resistance rates have been in the range of 20-30%. Almost similar success and relapse rates have been seen with single agent Arsenic Trioxide (ATO). Combination of ATRA with ATO as first line therapy is an attractive treatment option in APL. Both drugs have distinct action on degradation of PML-RAR alpha and thus act synergistically. *Methods.* All newly diagnosed patients with APL were given the option of combined therapy with ATRA and ATO after informed consent. This protocol was also used in those patients who had poor performance status and did not give consent for standard chemotherapy. The diagnosis of APL was made by bone marrow examination and PML-RAR alpha detection by RT-PCR. ATRA was used in the dose of 25 mg/m^2 and ATO in the dose of 0.15 mg/kg/day until complete hematological remission. Consolidation therapy consisted of ATRA for 6 weeks and 28 injections of ATO (5 days/week). All patients received three consolidation therapies each after a gap of one month. Subsequently after documentation of molecular remission, patients were put on maintenance chemotherapy consisting of intermittent ATRA 15 days every 3 monthly, 6-mercaptopurine 50 mg/m^2 daily and methotrexate 20 mg/m^2 weekly for 2-years. RT-PCR for PML RAR alpha was done after successful induction, after completion of consolidation and yearly during maintenance therapy. *Results.* From January 2005 till December 2009, 25 patients (13 males, 12 females) were offered combined therapy with ATRA and ATO. The median age of the patients was 33 years (range 13-48 years). The presenting complaints were low-grade fever and fatigue with median symptom duration of 30 days (range 5-90 days). Baseline median hemoglobin value was 7 gm/dl (range 6-12.4 gm/dL). White cell count of more than 10,000/μl was found in 12 patients (48%) and 21 patients (88%) had platelet count of less than 40,000/μL. Coagulopathy was present in 22 patients (85%). Hematological remission was achieved in 23 patients (92%) with median time to remission of 35 days (15-49 days). ATRA syndrome developed in 9 patients (36%). Two patients developed cardiac toxicity in the form of prolongation of QTc interval. Four patients died during therapy (two deaths during induction therapy, one after successful induction and one during maintenance therapy). One patient relapsed during maintenance phase. Two patients abandoned the treatment while in molecular remission. Remaining 18 patients are in molecular remission at a median follow-up duration of 17 months (range 3-57 months). *Conclusions.* Combination of ATRA and ATO produces high rates of successful induction in APL. The therapy is well tolerated without any major side effects and requires further evaluation in a randomized trial.

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IN ADULT ACUTE MYELOID LEUKAEMIA (AML) ALL RISK SUBSETS BENEFIT FROM A SEQUENTIAL HIGH-DOSE PROGRAMME AS EARLY RESCUE OF FIRST INDUCTION FAILURE: A REPORT FROM NORTHERN ITALY LEUKAEMIA GROUP (NILG)

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Background. In adult AML the complete remission (CR) rate following standard induction therapy varies according to incidence of risk factors. Because obtaining a CR is a primary determinant of survival, the analysis of induction outcome in function of risk group and specific treatment elements is essential to optimize induction results and eventually improve survival. *Aims.* To analyse (1) the failure rate to a standard induction regimen and (2) the salvage rate after a sequential high-dose programme in different risk subsets of adult AML, as defined by cytogenetics and selected additional risk factors. *Methods.* In prospective study NILG-AML 01/00, CR induction therapy consisted of standard ICE chemotherapy with idarubicin 12 mg/m^2/d dd 1-3, etoposide 100 mg/m^2/d dd 1-5, cytarabine 100 mg/m^2/bd dd 1-7, G-CSF from d 8. Refractory cases were to receive a sequential high-dose regimen with idarubicin 17.5 mg/m^2/d dd 1 and 8, cytarabine 3 g/m^2/bd dd 2, 3, 9, and 10, G-CSF from d 11 (s-HAI). Patients were stratified according to cytogenetics and additional risk factors (WBC count >50×10^9/L, FAB class M0/6/7, hepato/splenomegaly, MDS-related/secondary AML, FLT3/ITD mutation). Cytogenetic risk groups were favourable with t(8;21), inv(16); normal with 46 XX/XY; unfavourable with -5/del(5q), -7, abn(11q23), abn 3q/9q/11q/20q/21q/17p, iso(17q), t(3;5), t(6;9), t(9;22), complex with >3 unrelated clonal anomalies; and intermediate with other aberrations or unknown karyotype. Results. Between 2000-07, 581 patients were enrolled (age range 19-68 years, median 52). The distribution of cytogenetic abnormalities and the association with other risk factors is shown in the table. Overall incidence of hyperleukocytosis was 24%, adverse FAB class 10.2%, MDS/secondary AML 15%, hepato/splenomegaly 20%, and FLT3/ITD+ 27%. Following ICE induction, 22% of the patients had refractory AML. As expected, this figure varied greatly from favourable to unfavourable cytogenetic risk class (8% to 36.6%), with a nonsignificant trend to worse results conferred by additional risk factors in each cytogenetic risk group (cumulative resistance 24.3% *vs.* 18.7% without, P=0.1). Following s-HAI in 95/129 ICE-refractory patients (73.6%), the risk class effect was no longer detectable as demonstrated by the high cumulative CR rate (n=54, 56.8%), that was unrelated to cytogenetic risk subset and/or associated risk factors (Table). Conclusions. The integrated clinical and cytogenetic risk model identified patients at higher risk of resistance to standard ICE induction therapy. More than half of these cases were effectively rescued by s-HAI, regardless of cytogenetics or clinical risk class.

This documents the potential relevance of a combined risk classification and points to the inadequacy of conventional first-line therapy in HR subsets.

Table 1.

		cycle 1 (ICE)		cycle 2 (s-HAI)	
Cytogenetic risk class	Additional risk factors	No. treated	Refractory, no. (%)	Refractory treated, no.	Complete remission, no. (%)
Favourable	Absent	30	3 (10)	2	2 (100)
	Present	20	1 (5)	1	1 (100)
Normal	Absent	94	13 (14)	9	5 (55.5)
	Present	178	33 (18.5)	22	15 (68)
Intermediate	Absent	53	12 (23)	10	6 (60)
	Present	86	23 (27)	17	7 (41)
Unfavourable	Absent	42	13 (31)	8	5 (62.5)
	Present	78	31 (40)	26	13 (50)

0650

TREATMENT-RELATED AML AND AML EVOLVING FROM MDS: SIMILAR OUTCOMES FOLLOWING TREATMENT WITH AMONAFIDE + CYTARABINE

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Background. Secondary AML (sAML) is a well recognized poor prognostic factor in AML. sAML is a heterogeneous group of diseases, including both AML evolving from myelodysplastic syndromes (MDS, MDS→AML) and AML following chemotherapy for another malignancy, treatment-related AML (tAML). These two subtypes of poor risk AML have similar outcomes when treated with standard anthracycline-based chemotherapy, supporting their inclusion into the same clinical category. It is unknown whether this classification is valid when using experimental antileukemic agents. We therefore examined the outcomes of MDS→AML *vs.* tAML, following amonafide-based therapy. *Methods.* Patients (pts) received amonafide 600 mg/m^2/day IV days 1-5 and cytarabine 200 mg/m^2/day IV continuous infusion days 1-7. Post-remission therapy (PRT) included hematopoietic stem cell transplant (HSCT; n=10) or cytarabine (intermediate-dose, n=13; or high-dose, n=7). The primary endpoint was CR/CRi; secondary endpoints were safety, DOR and OS. Cytogenetic classifications were based on modified SWOG-ECOG criteria. *Results.* 88 pts with sAML were treated. 45 (51.1%) had tAML (including 5 pts with MDS→AML and tAML), and 48 (53.4%) had MDS→AML. Baseline characteristics were as follows: tAML: 12 (27%) male, age 60 yrs (23-87), 35 (78%) ECOG 0-1, 37 (86%) unfavorable cytogenetics; MDS→AML: 29 (67%) male, age 66 yrs (24-81), 37 (86%) ECOG 0-1, 15 (35%) unfavorable cytogenetics. 37 pts (42%) achieved CR+CRi. CR+CRi rate was 18/45 (40%) for tAML pts and 19/43 (44.2%) for those with MDS→AML (P=0.691). PRT was given to 16/18 tAML pts (89%, HSCT 5, HiDAC 4, IDAC 7), and 14/19 MDS→AML pts (74%, HSCT 5, HiDAC 3, IDAC 6). Median DOR: 512 days for tAML, 164 days for MDS_AML (P=0.026) Median OS: 209 days tAML, 183 days MDS→AML (P=0.091). *Conclusions.* tAML and MDS→AML are diseases with similar outcomes in response rate and overall survival following amonafide therapy, further supporting their inclusion together into a broad category of sAML, though duration of remission appears shorter for MDS→AML . These data validate sAML as an appropriate grouping when considering treatment options for these poor-risk patients.

0651

WT1 MUTATION IN 470 ADULT PATIENTS WITH ACUTE MYELOID LEUKEMIA-STABILITY DURING DISEASE EVOLUTION AND IMPLICATION OF ITS INCORPORATION INTO A SURVIVAL SCORING SYSTEM

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Background and *Aim.* The impact of WT1 mutations in acute myeloid leukemia (AML) is not completely settled. We aimed to determine the clinical implication of WT1 mutation and its stability during the clinical course. *Methods* and Materials. From November 1995 to March 2007, a total of 470 adult patients ≥15 years who were newly diagnosed as having *de novo* non-M3 AML at National Taiwan University Hospital (NTUH) were enrolled. Mutation analysis of WT1 exons 7 and 9 was performed by genomic DNA polymerase chain reaction (PCR) and was correlated with other gene mutations and clinical features. Results. WT1 mutations were identified in 6.8% of total patients and 8.3% of younger patients with normal karyotype (CN-AML). The WT1 mutation was closely associated with younger age (P=0.0008), FAB M6 subtype (P=0.006), and t(7;11)(p15;p15) (P=0.0025). Multivariate analysis demonstrated that WT1 mutation was an independent poor prognostic factor for overall survival and relapse-free survival among total patients and CN-AML group. A scoring system incorporating WT1 mutation with FLT3/ITD, NPM1 and CEBPA mutations into survival analysis was proved to be very useful to stratify CN-AML patients into different prognostic groups (P<0.001). Sequential analyses were performed on 133 patients. WT1 mutations disappeared at complete remission in all WT1-mutated patients studied. At relapse, three of the 16 WT1-mutated patients who had paired samples lost the mutation and two acquired additional mutations, while three of 110 WT1-wild patients acquired novel mutations. *Conclusions.* WT1 mutations are correlated with poor prognosis in AML patients. The mutation status may be changed in some patients during AML progression.

0652

BIPHENOTYPIC ACUTE LEUKAEMIA: TREATMENT WITH ALL-TYPE REGIME MAY OFFER A BETTER OUTCOME

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Background. Biphenotypic acute leukemia (BAL) is an uncommon disease and accounts for 4% of acute leukaemias. There is no standard treatment. *Aims.* We present an overview of the clinical and biological characteristics, response to treatment and outcome of BAL in a single institution over 8 years. *Methods.* BAL patients were identified through a search of the Leukemia Registry (a computerized database approved by the Ethics committee). Informed consent was waived by EC. The characteristics and clinical profile of patients diagnosed with BAL according to European Group for the Immunological Characterisation of Leukemias (EGIL) Classification were studied. Results. BAL was diagnosed in 22 patients (12 males and 10 females) amongst 442 acute leukemia cases between 1998 and 2006. BAL accounted for 5% of acute leukemias. Median age was 42 years. Eight patients co-expressed B-lymphoid/myeloid antigens while 14 patients co-expressed T-lymphoid/myeloid antigens. No patient demonstrated trilineage differentiation. Cytogenetic analysis revealed normal karyotypes in 6 patients (27.3%) and abnormal karyotypes in 16 patients (72.3%). Four patients carried the Philadelphia chromosome (Ph) t (9; 22), while 2 patients had karyotype involving chromosome 11. Two patients received ALL type induction therapy, while 17 patients received combined ALL and AML type induction therapy. Complete remission (CR) was achieved in both patients (100%) that received ALL type induction therapy over a mean period of 1.5months. One patient is alive after 10 months from diagnosis and has received allogenic bone marrow transplant. In the group that received combined ALL and AML type induction therapy, CR was achieved in 8 patients (47.1%) over a mean period of 1.4 months, however, 4 patients relapsed after a mean period of 6.8 months and died. The remaining 4 patients in CR received allogenic bone marrow transplant. Two patients are alive after a mean period of 78.5 months from diagnosis while 2 died after a mean period of 8.5months from CR. Summary/Conclusions. In this study, the incidence of T-lymphoid/myeloid leukemia was 63.6% while that of B-lymphoid/myeloid leukemia was 36.4%. Cytogenetic analysis revealed a high incidence of chromosomal abnormalities; 18.2% displayed the Ph chromosome while 9.1% had chromosome 11 abnormalities. Both are poor prognostic markers where 5 patients died and only 1 patient with the Ph chromosome is still alive after 10 months, having received ALL type induction therapy, imatinib and allogenic bone marrow transplant. Induction therapy with ALL type regime resulted in a disease free survival (DFS) greater than 6 months in both patients. Of the 4 patients that remained in CR after combined induction therapy, 50% died within 6 months, while 50% were still alive after a mean period of 78.5 months. No firm recommendations can be made regarding remission induction therapy as numbers are small. Patients receiving ALL type induction therapy seemed to fared better. At our centre, newly diagnosed BAL patients are now treated with ALL type induction therapy. For treatment protocols to be established in this rare disease, multi-center studies are required.

0653

OUTCOME OF THERAPY-RELATED ACUTE MYELOGENOUS LEUKEMIA AND MYELODISPLASTIC SYNDROMES RECEIVING ALLOGENEIC TRANSPLANTATION: THE IMPACT OF PERFORMANCE STATUS, CYTOGENETICS AND PREVIOUS TREATMENTS

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Backgroud. Allogeneic stem cell transplantation (alloSCT) can cure few patients with therapy-related myelodisplastic syndromes (tMDS) and acute myelogenous leukemia (tAML). Disease- and transplant-related mortality remain very high despite the use of less intensive conditionings. *Aims.* This retrospective study evaluated which characteristics may affect the survival outcome of tMDS/tAML patients receiving alloSCT. *Methods.* All the 28 patients with tAML (86%) or tMDS (14%) allografted in 4 Italian centers between 1998 and 2009 were considered eligible. Statistic methods included Kaplan-Meier analysis of overall (OS) and progression free survival (PFS), and Cumulative Incidence analysis of non-relapse mortality (NRM) and relapse incidence (RI). Patients' median age was 49 years (range 21-65), previous neoplasia was Hodgkin's and non-Hodgkin's lymphoma (54% and 29%, respectively), and non-hematologic cancer (17%). Treatment of previous cancer included chemotherapy (31%), radiotherapy (14%) or both (55%), 36% of patients received >2 lines of therapy before the onset of tMDS/tAML. t-AML (86% of patients) or tMDS (14%) was diagnosed at a median time of 86 months (range 13-253) from previous cancer. Twenty-four patients (86%) had cytogenetic analysis available, 33% of them had intermediate-risk and 67% high-risk cytogenetics according to Medical Research Council AML 10 Trial definitions. Twenty-three patients (82%) received induction, 20 (71%) consolidation chemotherapy. At transplant, 40% of patients was in CR1, 3% in CR2, 14% in PR and 25% in PD, 18% of patients received upfront alloSCT due to low blast percentage. Median time from tMDS/tAML diagnosis to alloSCT was 5.7 months (range 0-25). At transplant, 9 patients (32%) had a Karnofsky Performance Status (KPS)<=80%. Patients underwent reduced intensity (RIC, 39%) or myeloablative (61%) alloSCT from HLA identical (32%) or mismatched siblings (3%), unrelated (54%) or haploidentical donors (11%). Results. Twelve patients (43%) are alive at last follow-up, 7 (25%) died of disease, 9 (32%) died of NRM. Median follow-up of surviving patients was 528 days (55-1704). One- and 2-years OS was 50% and 36%, 1- and 2-years PFS was 42% and 38%. RI was 28% at both 1 and 2 years, NRM was 18% at 100 days, 30% at 1 year and 35% at 2 years. OS and PFS were significantly affected by KPS<=80% (P=0.008 and P<0.001, respectively) and high-risk cytogenetics (P=0.03 and P=0.01). RI was reduced by KPS>80% (P=0.02), chemo-responsive disease (trend, P=0.05) and intermediate-risk cytogenetics (trend, P=0.08). Treatment of previous neoplasia with >2 lines of therapy was correlated with a worse NRM (P=0.005) and a worse OS (trend, P=0.05), whereas the type of treatment (radiotherapy+chemotherapy *vs.* chemotherapy *vs.* radiotherapy) did not affect survival, NRM and RI. NRM was increased in patients who had infectious complications after induction (P=0.009) or received consolidation therapy (P=0.03), and in patients allografted >180days after tMDS/tAML (trend, P=0.05). The type of conditioning (RIC *vs.* myeloablative) did not affect survival, NRM and RI. Conclusions. In conclusion, some tMDS/tAML patients undergoing alloSCT can survive leukemia-free at 2-years. Patients' selection should be based on the assessment of KPS, cytogenetics and treatment history before alloSCT. Prospective trials are awaited in order to confirm these results.

0654

TREATMENT OF ACUTE MYELOID LEUKEMIA IN OLDERS WITH AN OUTPATIENT FLAG-IDA PROTOCOL

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State of the art. Acute myeloid leukemia (AML) is associated with poor prognosis in older patients and there is no a truly adequate treatment. Results of standard chemotherapy are dismal. Adverse biology and poor tolerability by their poor performance status and their significant comorbidities are the causes for bad results. Thus, new treatment options are necessary. We present the results of a new chemotherapy outpatient protocol in older patients (>65 years) with AML. Study design. Patients were assigned to receive: G-CSF (subcutaneous, 300 ug days -1 to 5), Fludarabine (oral, 40 mg/m^2 days 1 to 5), Cytarabine (subcutaneous 20 mg/m^2 days 1 to 5) and Idarrubicin (oral, 15 mg/m^2 days 1 to 3) as induction (1-2 cycles) and consolidation (2 cycles) therapy. Inclusion criteria were age >65 and significant associated comorbidity. If possible, they were followed as outpatients. Patients characteristics. Between 2006 and 2009, 30 patients with a median age of 78 years (66-91 years) were enrolled. Twenty-nine (97%) showed significant associated comorbidity [heart disease (58%) or lung disease (26%)]. ECOG score was 0-1 in 26 patients. There were 25 AML [(15 *de novo* and 10 secondary to myelodysplastic syndrome (MDS)] and 4 RAEB-2. Overall, 20% of patients had an unfavourable karyotype and 53% an intermediate karyotype. Median WBC was 4550/uL (range: 500-192000). *Results.* Treatment was well tolerated and followed in outpatient management in 73%. Twenty-six patients (86%) received a unique induction cycle and four needed to receive two cycles. Overall response rate was 57% [complete response (CR) in 50% and partial response (PR) in 7%]; 33% were refractory. The median to neutrophil recovery (>500 PMN) was 22 days for responders. Two patients died in induction. Fourteen patients got to receive consolidation therapy. A total of 62 cycles were administered. During induction cycles 40% required temporary hospital admission due to febrile neutropenia/pneumonia (9 patients), bleeding (2 patients) or bradyarrythmia (1 patient). Hospital admission was not necessary in any patient during consolidation cycles. Twenty-one patients died (17 by leukemia progression and 4 by treatment-related complications). Nine patients remained in CR (2 underwent an RIC allogeneic SCT in first CR). Overall survival at 40 months is 30%. Adverse factors related with survival were unfavourable karyotype, secondary AML and refractoriness to treatment. *Conclusions.* FLAG-IDA protocol in older patients with AML is well tolerated and is an excellent therapeutic option with a significantly higher survival in responders. Moreover, it offers the advantage of outpatient management, improving quality of life.

0655

MONOSOMY KARYOTYPE IN ACUTE MYELOID LEUKEMIA PREDICTS ADVERSE TREATMENT OUTCOME WITH LOW COMPLETE REMISSION RATE AND WORSE EVENT-FREE AND OVERALL SURVIVAL, AND ASSOCIATES WITH HIGH FUNCTIONAL ACTIVITY OF P-GLYCOPROTEIN, MDR1

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Background. It has been revealed that monosomy karyotype (MK), defined as 1) two or more distinct autosomal chromosome monosomies or 2) one single autosomal monosomy in the presence of structural abnormalities, identifies the highly unfavorable cytogenetic risk group of patients with acute myeloid leukemia (Breems, J Clin Oncol 2008), but lacking validation data. *Aims.* The current study aimed to validate the unfavorable impact of MK not only on overall survival (OS) but also on complete remission (CR) rate and event free survival (EFS) in AML patients. *Methods* and patients. A total of 370 consecutive AML (excluding APL) patients with available cytogenetic data who received treatment between 1995 and 2008 at the Samsung Medical Center, Seoul, Korea were included in this retrospective study, among whom 169 patients (45.7%) showed normal karyotype; 65 patients (17.6%),

core binding factor (CBF) positive AML; and 136 patients (36.7%), non-CBF AML, respectively. Karyotypes were scored according to their structural abnormalities, monosomy, trisomy, deletion and marker chromosome. The CR rate, EFS and OS were compared according to the presence of each cytogenetic abnormality. In addition, multidrug resistance (MDR) functional assay (P-glycoprotein assay) was performed and MDR functional activity was calculated by verapamil-inhibited rhodamine-123 efflux activity minus uninhibited rhodamine-123 efflux activity, and positivity was determined as equal to or over 5% MDR activity. *Results.* Among treated non-CBF AML group with any kind of cytogenetic abnormalities (n=136), 95 patients (69.9%) had structural cytogenetic abnormalities, 29 pts (21.3%), autosomal monosomy, 18 pts (13.2%), sex chromosome abnormalities, 59 pts (43.4%), autosomal trisomy, 41 pts (30.1%), deletion of part of a chromosome, 18 pts (13.2%), addition and 18 pts (13.2%), marker chromosome(s). MK was noted in 23 patients (16.9%), and complex karyotype (≥3 abnormalities) were found in 40 pts (29.%), -5 in 5 pts (3.7%), -7 in 12 pts (8.8%), del(5q) in 4 pts (2.9%), del(7q) in 8 pts (5.9%), inv(3) or t(3;3) in 4 pts (2.9%), t(6;9) in 5 pts (3.7%), and t(9;22) in 2 pts (1.5%). In univariate analyses, MK group was revealed to be associated with shorter OS and (median 10 *vs.* 31months, P=0.044) EFS duration (median 1.3 *vs.* 1.3 months, P=0.002), and a lower CR rate (70.8% *vs.* 34.8%, P=0.002). In a multivariate analysis, MK was associated with lower CR rate (HR of non-CR 0.33, 95% C.I. 0.12-0.93, P=0.036). MK has been defined as a single monosomy with structural abnormalities or multiple monosomies in a previous study. However, there were no significant difference in survival and CR rate between a single monosomy with (n=9) or without(n=6) structural abnormalities (OS, 23 *vs.* 8 months; P=0.349; EFS, 1 *vs.* 9months; P=0.078; CR rate 33% *vs.* 56%; P=0.608). The group with single autosomal monosomy showed a trend of better survival (n=15, median OS 23 months, EFS 1month) than multiple autosomal monosomy group (n=14, OS 6months, EFS 1month), but it was not significantly different (P=0.322, P=0.221). The functional MDR activity was measured in 40 patients, and positive MDR activity was found to be significantly associated with the presence of MK (87.5% *vs.* 33.3%, P=.013). In addition, the functional MDR activity was significantly higher in MK+ group (n=8, 45.9±17.8%, mean±S.E.) than in MK- group (n=32, 4.3 ±2.7%, P=0.005 by Mann-Whitney U-test). *Conclusion.* The current study demonstrated that the AML patients harboring MK showed a poor outcome in terms of lower CR rate and worse EFS/OS in an independent cohort of Korean AML patients, and that MK karyotype was associated with high MDR functional activity of leukemic blasts.

0656

ALLOGENEIC HAEMOPOIETIC STEM-CELL TRANSPLANTATION USING A NON T-CELL DEPLETE REDUCED INTENSITY CONDITIONING PROTOCOL FOR ACUTE MYELOID LEUKAEMIA & MYELODYSPLASIA: A 10-YEAR SINGLE CENTRE EXPERIENCE

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Background. Over the last decade a number of reduced intensity conditioning (RIC) protocols have been developed, for treatment of patients with acute myeloid leukaemia (AML) and myelodysplasia (MDS). RIC haemopoietic stem cell transplants (HSCT) utilise the power of the graft *vs.* leukaemia effect and result in a lower transplanted-related mortality than standard myeloablative HSCTs. These protocols can be categorised into T-cell deplete (TCD) and non-TCD. The use of T-cell depletion results in less chronic graft *vs.* host disease (GvHD) but, in view of the impaired immune reconstitution, results in an increased risk of infections, particularly CMV re-activation, and potentially increased relapse risk. *Aims & Methods*. A retrospective analysis was performed on the outcomes of the RIC transplant programme at St. Bartholomew's Hospital between April 1999 and July 2009. Fifty consecutive patients, for whom a conventional transplant was deemed inappropriate, received a non-TCD RIC allogeneic (HSCT) for treatment of AML and MDS. The median age of the patients was 53 years. Thirty-eight patients were diagnosed with AML and 12 with MDS. Patients received fludarabine 25 mg/m^2 for 5 days (day -6 to day -2) and cyclophosphomide 1g/m^2 for 2 days (day -3 to day -2) as the conditioning schedule. Results. Median follow up of surviving patients was 42 months. The 5-year predicted overall survival (O/S) and event-free survival (EFS) was 56% and 52% respectively and the non-relapse mortality (NRM) was 6%. Acute and chronic GvHD incidence was 14% and 41% respectively. Occurrence of chronic GvHD was predictive of improved outcome, with 5-year O/S and EFS of 84% and 86% compared to 20% and 31% for those with and without chronic GvHD. Furthermore, 88% of patients with chronic GVHD were off immunesupression at 2 years follow up. Disease relapse occurred in 46% of patients that also included 3 cases of late extramedullary relapse. Systemic relapse occurred early and all within 1-year of follow up. However, due to the low toxicity of the conditioning protocol, a cohort of patients was able to undergo a further RIC transplant procedure. Summary. In conclusion, we present long-term follow-up data on a single centre experience of a non-TCD RIC allogeneic allograft regimen for patients with AML and MDS. This protocol is associated with a very low NRM, low levels of acute GvHD and good overall survival rates. Chronic GvHD, although more frequent than in TCD allograft procedures, is associated with a profound GVL effect and predictive of long term overall survival.

0657

THE CLINICAL CHARACTERISTICS AND PROGNOSIS OF ELDERLY PATIENTS WITH ACUTE MYELOGENOUS LEUKEMIA IN KOREA; THE PROPOSAL OF NOVEL PROGNOSIS SCORING SYSTEM FOR ELDERLY PATIENTS

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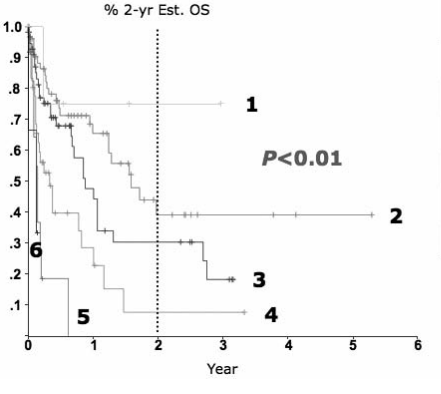
Background. Acute myelogenous leukemia (AML) is mainly a disease of older adults. Benefit of standard cytotoxic therapy for elderly patients remains debated because of excessive toxicity and short response duration. So, an organized and multi-disciplinary approach has to be required for these population, and especially, identification of subgroups who might benefit from chemotherapy is important. *Aims.* 1, to understand clinical characteristics of elderly patients with AML in Korea. 2, to analyze clinical outcomes, including response to chemotherapy. 3, to identify factors predicting responses to specific treatment. 4, to establish composite prognostic scoring system for determining personalized treatment strategy in elderly AML. *Methods.*From 17 institutes in Korea, 614 elderly (>60 yo) AML patients were enrolled and evaluated retrospectively by CRF collection.



KAMWP Elderly AML model	
Score	%2-yr Est. OS
1	75.0
2	39.1
3	30.2
4	7.6
5~6	0

Figure 1. KAMDS score.

Results. The mean age was 68.9 (60~101) and male to female ratio was 1:28:1. Twelve percents of patients had preceded hematologic diseases.

M2 and M4 by FAB classification were most common subtypes. Unfavorable cytogenetics were documented in 24% of patients. Remission induction chemotherapy was performed for 63% of patients and complete remission rate was 60.7%. Three year overall survival rate was significantly higher in treated patients (30.7% *vs.* 6.9%, P<0.0001), and relatively younger (60<<75) patients and patients with lower ECOG score showed higher survival rate. We made new prognosis scoring system using age, ECOG, initial WBC count, cytogenetics and myelofibrosis. By this scoring system, 2 year overall survival rate of elderly patients were significantly differentiated (Figure). Summary/*Conclusions.* Our novel scoring system using 5 factors in this study may be feasible and optimal for elderly patients. Furthermore, this scoring system need to be validated with prospective studies with larger number of patients and different races.

0658

DAY 15 BONE MARROW DRIVEN DOUBLE INDUCTION IN YOUNG ADULT PATIENTS WITH ACUTE MYELOID LEUKEMIA: FEASIBILITY, TOXICITY AND THERAPEUTIC RESULTS

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Background. The strategy named double induction (DI) in patients with acute myeloid leukemia (AML) consists of two courses of chemotherapy irrespective of the degree of cytoreduction in the bone marrow after the first course, with the second course starting on day 21 or on day 11 to 12 unless severe complications prohibit its application. *Aims.* To describe disease characteristics and treatment results from a series of 33 patients with AML, in whom day 15 (D15) morphological and immunophenotypic examination of bone marrow revealed persistence of more than 10% blast cells and therefore candidate to receive double induction with an additional course of chemotherapy. *Methods.* The median age was 50 years (16-66). All patients receive as induction the ICE regimen [idarubicin 10 mg/ sqm on days 1, 3 and 5; cytarabine (ARA-C) 100 mg/sqm as continuous infusion (c.i.) on days 1-7; etoposide 100 mg/sqm on days 1-4]. As second induction, we administered the combination of fludarabine (F), intermediate dose cytarabine (ARA-C) and G-SF. More in detail, F was given at 30mg/m2/day as 30 min intravenous perfusion and ARA-C 2 g/m^2/day 3 hour and half after the end of F from day 1 to 5. G-CSF at 300 mg was concomitantly given from day 1 up to CR achievement. *Results.* The median blast count at D15 was 30 (15-90). Overall, 30 out of 33 patients were judged as eligible to receive DI. Reasons for exclusion in three patients included in all cases active pulmonary infection (bacterial in two and fungal in one patient) in the context of severe pancytopenia. Of note, all patients were severely neutropenic at day 15 (< 0.5×10^9/L). According to cytogenetic examination, 19 patients (63%) had unfavorable karyotype and 11 (37%) showed diploid karyotype; 6 of these had FLT3/ITD mutation, while NPM1 mutation was found in 1 patient in association with FLT3/ITD. Overall, CR was achieved in 20/30 patients (67%), while 8 patients (27%) were classified as refractory, and 2 died of infectious complications while severely cytopenic (6%). Of interest, all refractory patients had unfavorable cytogenetics. The median number of days from start of DI to neutrophils > 0.5×10^9/L and platelets > 20×10^9/L was 22 and 30, respectively. Patients received a median of 13 packed red cells (range: 10-18) and 19 platelet units (range: 13-26). All patients achieving CR were programmed to receive allogeneic stem cell transplantation (allo-SCT), which was actually performed in 11 patients. On the contrary, allo-BMT was not performed in 9 patients because of early relapse in 6 patients, refusal in 2 and loss to follow-up in 1. Summary and *Conclusions.* Our study suggest that D15 driven DI represents a feasible and effective therapeutic strategy in young adult AML patients. Although inducing more relevant hematologic toxicity and longer hospitalization than conventional induction, DI improves therapeutic results and do not compromise feasibility of allo-SCT. Finally, as compared to conventional DI, day 15 driven DI offers the potential to avoid unnecessary toxicity in a consistent proportion of patients.

0659

ATRA AND ANTHRACYCLINE BASED CHEMOTHERAPY IN CHILDHOOD ACUTE PROMYELOCYTIC LEUKEMIA (APL): 10 YEAR EXPERIENCE WITH TWO SUCCESSIVE PROTOCOLS IN 20 PATIENTS

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Background. The combination of ATRA and intensive chemotherapy is very effective in childhood APL, a rare disease. Reports on childhood APL from developing countries are scarce. We report results of a 10 year experience in management of childhood APL and compare them with results obtained with adult APL in the same period. *Methods.* 65 genetically confirmed APL, either by t (15, 17) and/or PML/RARA, were treated with two consecutive protocols: 24 (36.9 %) with The European APL 93 (1998-2004) and 41 (63.1%) with the Spanish LPA 99 protocol (from 2004). Twenty (30.7%) were aged less than 20 years. In the same period 45 adults were treated with APL93 (n=18) and LPA99 (n=27) trials. *Results.* The 20 children included 11 girls and 9 boys; median age was 12 years (range, 4-19 years). Fever as initial symptom was more frequent among children (16/20) than adults (17/45) (P=0.002). 13 children (65%) had classical APL and 7 (35%) had variant APL, compared to 39 (87%) and 6 (13%), respectively, in adults (P=0.044). Additional cytogenetic abnormalities were observed in 3 children and 14 adults (P=0.14). According to Sanz'score 1 (5%) child had low risk, 11 (55%) intermediate and 8 (40%) high risk (compared to 7%, 60% and 33%, respectively of adults: P=0.6). During induction course differentiation syndrome (DS) diagnosed according to Frankel's criteria (Ann Intern Med 1992; 117: 292-6) was less often observed in children (1/20) than adults (13/45) (P=0.031, RR=0.21). The CR rate was 95% (19/20) in children and 80 % (36/45) in adults (P= 0.13). Two children relapsed and died during salvage therapy, 1 patient died in CR from infection during the third consolidation course and the last patient, aged 8 years, died from cardiac failure 15 months after CR (without any other cause of cardiac disease, and with a cumulative dose of idarubicin of 90 mg/m^2 and mitoxantrone of 50 mg/m^2) leading to a 4 year EFS of 78.9% compared with 88.9% in adults (P=0.44). With a median follow-up of 4 years, OS was 75% in children *vs.* 77.8 % in adults (P=0.94). Age (P=0.9), sex (P=0.19) and baseline WBC (P=0.29) had no impact on survival in children. *Conclusion.* We found comparable outcome in children and adult APL. Of note was the significantly lower incidence of differentiation syndrome in children (compared to adults) and the fact that one patient treated died from cardiac failure attributed to anthracyclines, a possibly emerging problem with high cumulative doses of anthracyclines in childhood APL.

0660

ROLE OF AGE AND CYTOGENETIC DATA IN THE OUTCOME OF FIT ELDERLY AML PATIENTS

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Background. Acute Myeloid Leukemia (AML) is most common in the elderly and the patients are often thought to be unfit for chemotherapy. Patients with poor prognostic karyotype are even not recommended for treatment outside clinical protocols. In this context prognostic factors are important tools for clinical judgement, decision making and patient information. Objectives. We retrospectively reviewed whether this assessment is validated in our AML population older than 60 yo. Population and methods. 82 AML patients, 60 yo and older, were referred to our centre between 1987 and December 2009. Patients requiring treatment were treated according to the EORTC (LAM 13 and LAM 17) protocols for patients above 60 years old. AMLs were classified according to the old FAB classification. Cytogenetic data were obtained by routine karyotype and additional FISH analysis since 1995. Complete remission was defined by morphology and flow cytometry on bone marrow smear. Karyotypes were stratified according to good and intermediate 1 *vs.* intermediate 2 and poor prognosis (ELN recommendation). Statistical analyses using age and Karyotype were performed for the following outcomes: complete remission (CR), overall survival, median survival and disease-free survival. *Results.* 76 files were evaluable for cytogenetic data and outcome. Median age was 70 (60-

86) years old. Because we are a referral tumour centre, all the patients had a PS <3 and no geriatric syndrome (falls, dementia, incontinence). The median follow up time was 40 (1-180) months. Overall survival for the whole population was 17% at 180 months with a median survival of 7 months. Median survivals of patients below 70 years old was significantly better (P<0.0001) than patients above 70 years old (38 *vs.* 7 months). Whatever the age, median survival was significantly better for patients in CR after the induction (48 *vs.* 6 months) and presenting with a favourable Karyotype (35 *vs.* 9 months) . Taking into account the cytogenetic data and age, the median survival of AML patients below 70 yo with a favourable Karyotype was 64 months with 40% of CCR, very similar to the younger population. Conclusion. in our series of fit elderly AML patients, we confirm that cytogenetic data have a major impact of OS and CCR in patients between 60 and 70 years old. For patients above 70 years old, median survival remains unsatisfactory and these patients should be offered new alternative treatment approaches.

0661

THE IMPACT OF CD117 (C- KIT GENE EXPRESSION) ON APOPTOSIS AND SURVIVAL IN ADULT ACUTE MYELOID LEUKEMIA(AML) PATIENTS

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Introduction. The proto-oncogene c-kit encodes a trans-membrane receptor belongs to class III receptor tyrosine kinase family involved in development of hemopoietic cells and leukemogenesis supporting apoptosis resistance in leukemia blasts. The aim of this study is to evaluate CD 117 expression together with the apoptosis of the myeloid blast cells in adult patients with AML and correlating them with other prognostic parameters and treatment outcome. Patients and *Methods.* forty seven patients with newly diagnosed AML were included. Clinical examination and laboratory investigations including bone marrow aspiration for morphological examination, immunophenotyping and cytogenetic assay were done.CD117 and apoptosis (annexin V)quantification by flowcytometry were done before and after the induction therapy. Results.The median age of patients was 44±15years.15/47≈32% had unfavorable cytogenetics,16/47 ≈34% had favorable cytogenetics and the rest were intermediate risk.CD 117 was positive in 20/47 ≈43% before induction, patients with +ve CD 117 were significantly older than patients with -ve CD 117(49±15 *vs.*40±13 years respectively p value=0.02). At presentation, the percent of apoptotic blasts was lower in CD 117 +ve patients (24%) than CD117-ve patients (30 %) but the difference was not significant. There was no significant correlation between CD117 positive blasts (% and or mean fluorescence index MFI) and apoptotic blasts (% and or MFI) neither at presentation nor after treatment. Highly significant negative correlation was found between CD117 +ve blasts and CD57 (natural killer cells) before and after treatment, with a significant positive correlation between CD117 MFI and the number of infection episodes during induction and follow up period. CD 117 MFI at presentation was significantly inversely correlated to overall survival with p value 0.04. The percent of apoptotic blasts at presentation positively correlated with overall survival p value 0.02. *Conclusion.* c-kit gene expression detected by CD117 phenotype and lesser apoptotic blasts at presentation harbor a worse prognosis for AML patients. Targeting c-kit gene is a promising potential in therapy of c-kit positive AML elderly patients.

0662

ABERRANT GENE EXPRESSION PROFILE OF ADULT DE NOVO ACUTE MYELOID LEUKEMIA WITH T(8;21)

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Background. Acute myeloid leukemia (AML) with t(8;21) was usually considered as a favorable subtype leukemia. However, it is highly heterogeneous in clinical practice. A lot of parameters including clinical features, cytogenetics and immunophenotypes were investigated, nevertheless, their clinical significance were still paradoxical. Aberrant gene expressions such as c-kit mutations have been considered as the poor prognostic factors in t(8;21) AML. Recently, more aberrant gene expressions including JAK2, FLT3 and TET2 have significant prognostic values in myeloid malignancies. *Aims.* To investigate the prognostic significance of JAK2, FLT3, c-kit and TET2 mutations in AML patients with t(8;21). Methods. The information including clinical features, cytogenetics, immunophenotypes, treatments and overall survivals were collected. JAK2, FLT3, c-kit and TET2 mutations were analyzed according to the published literatures. *Results.* Fifty-nine AML patients with t(8;21) were enrolled in the study. The mutant cases of c-Kit, tet2 and FLT3 were 20(20/59, 33.9%), 8(8/59, 13.6%) and 3(3/59, 5%), respectively. No JAK2 mutation was detected in this group. The overall survival rate at 5 years in patients with c-Kit, tet2 and FLT3 mutations was inferior to the non-mutant patients. The lose of sex chromosome, additional chromosome alternations and CD56 expression in the group were unrelated to the patients prognosis. *Summary/Conclusions*. c-Kit, TET2 and FLT3 mutations were the adverse prognostic factors in AML with t(8;21), and allogenetic stem cell transplantation might improve the prognosis of these patients.

0663

RESULTS AND TOXICITY PROFILE IN ADULT AML PATIENTS TREATED IN FOUR CONSECUTIVE RUSSIAN MULTICENTER CLINICAL TRIALS

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Background. Since late 1980s results in adult AML (16-60 yy) excluding patients with APL did not change dramatically, constituting 30-40% of 5-years overall survival in the selected for clinical trials cohort of patients, but the intensification of treatment protocols within this period of time was substantial. Each dose escalation and drug combination approach is followed by increased toxicity - severe myelosupression, increased transfusions support and rising rate of infections, thus potentially decreasing the tolerability of treatment. *Aim and Methods.* We evaluated the efficacy and toxicity profile of the treatment protocols of 4 consecutive Russian multicenter clinical trials since Jan 1992 till Dec 2009, comprising 1137 pts with AML (median age - 39 y (16-60yy)) from 32 hematological centers. Pts with APL treated in first two trials and those lost from follow-up were excluded, so the outcome of 899 AML pts was analysed. The results of four trial are depicted in Table 1.

Table 1.

Trials AML-92 AML-95 AML-01.01 AML-06.06

Outcome	(n=185)	(n=251)	(n=354)	(n=109)
CR,%	63,4	61	65	78
CR after c.N1 %	51,9	48,6	55,4*	51
DR,%	15,1	21,9	34,1*	3
ED, %	21,5	17,1	10,5*	19
Platelet transfusions, doses	27	54	59	81
Death in CR,%	18,1	15,3	10	13
Fulfilled protocol, % pts	no data	no data	75%	25%
5 years OS / DFS, %	25/35	25/28	25,5/25	38/22 (3y)

**Parameters were evaluated only after the 1st induction course*

Within 4 trials CR rate after the 1st course was identical, after the 2nd induction - substantially increased in the last trial due to HDARA-C. The early death rate was high in all trials. The main death reason were infections. Infections were registered in 95% of pts during induction. During the analysed period pneumonias were diagnosed in 43,6%-52% pts, not differeing much among trials. The rate of sepsis and enterocolitis decreased from 29-46.5% to 19%, and 27-40.8% to 17,5%. In the last trial the incidence of invasive aspergillosis was 4,7% after the 1st induction and 6% - after the 2nd induction courses. Hemorrhage as one of the death's reasons deacreased from 35% to 24,4% due to the increasing plateletes support from 27 to 81 doses within two induction courses. The death rate in CR also decreased from 18,1% to 10-13%, but tolerability of the whole treatment and compliance worsened comparing AML-01.01 and AML-06.06. Only $^1/_4$ of pts in the last trial completed prescribed treatment due to poor tolerability of two HAM and 2 HDARA-

C as consolidation. The very intensive protocol AML-06.06 did increase CR rate, but worsened the comliance and survival results. In a multivariant analysis among common risk factors (age, cytogenetics) the number of randomised by each hematological center pts (<>10 pts for the trial) became significant factor of prognosis - less experience worser results. We conclude that RLSG treatment protocols should be balanced by toxicity profile and experience of the centers, and that the most important factor for improving results is the inclusion of pts into clinical trials.

0664

RESPONSE TO DIFFERENT TREATMENT SCHEDULES IN PATIENTS FAILING TO 400 MG OF IMATINIB. RESULTS FROM THE CML SPANISH REGISTRY (RELMC)

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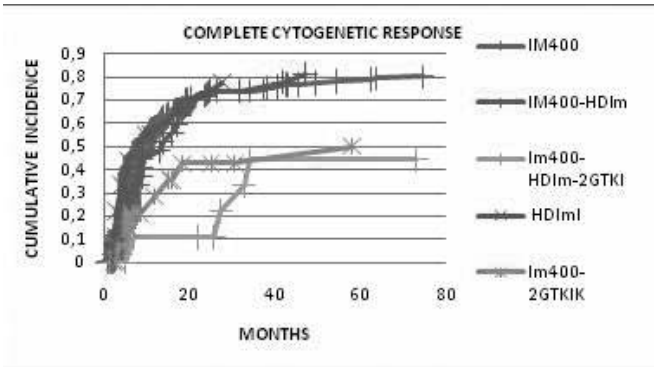
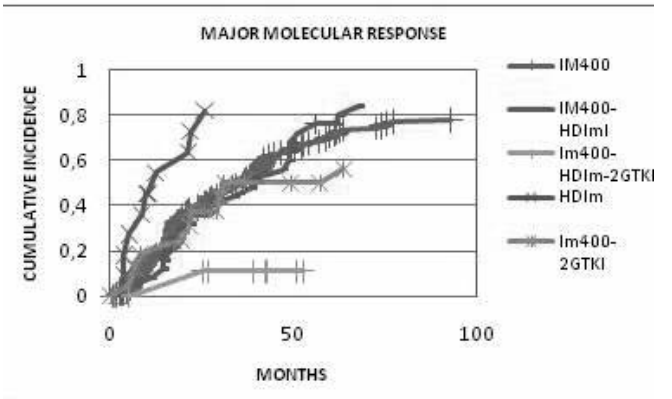
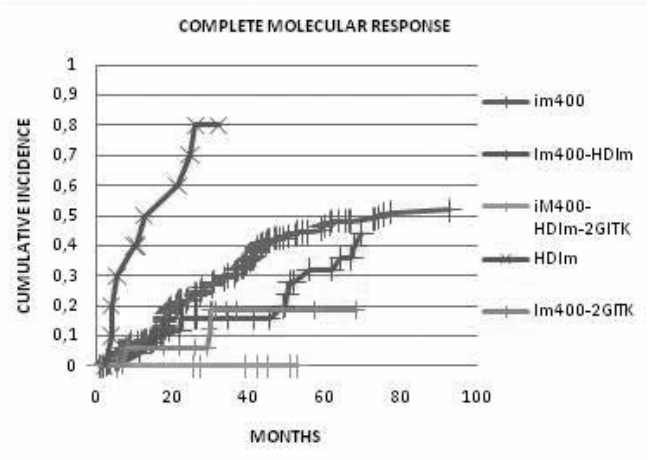
Background. The Spanish CLM Registry (RELMC) is a multicentric, hospital-based cancer registry which aim is to describe the actual treatment received by patients with CML in Spain, their outcomes, and the variables that influence treatment choices. There is no common treatment protocol. *Aim.* To study the best final cytogenetic, as well as the major and complete molecular response in 223 newly diagnosed CML patients treated with Imatinib as first line treatment that change treatment due to inadequate response to the initial treatment. During their evolution, the patients included in the Registry have received several lines of treatment: 1. Im400. 159 patients received Imatinib 400 only Im400-HDIm: 27Patients received Imatinib 400, followed by high dose Imatinib (600 or 800). 2. Im400-HDIm-2GTKI: 9 Patients received Imatinib 400, followed by high dose Imatinib (600 or 800), followed by 2nd generation TKI. 3. HDIm: 11 Patients received high dose Imatinib only. 4. Im400-2GTKI: 17 Patients received Imatinib 400, followed by 2nd generation TKI. Patients and *Results.* Two-hundred and twenty three newly diagnosed CML patients have been analyzed. A summary of response rates and cumulative incidence are included in Table 1.

Table 1.

GROUP 1	SOKAL	N	CCyR	MMR	CMR
Imatinib 400 MEDIAN FOLLOW UP 36,8 months N=159	LOW	77 (49%)	50/66 (76%)	54/65 (83%)	38/65 (58%)
	INTER	57 (36%)	43/52 (83%)	39/54 (72%)	28/54 (51,8%)
	HIGH	24 (15%)	20/23 (87%)	14/19 (73%)	6/19 (31,5%)
			NS	NS	NS
	HASFORD				
	LOW	72 (46%)	47/62 (76%)	48/62 (77%)	32/62 (52,2%)
	INTER	82 (52%)	63/76 (83%)	58/72 (80%)	40/72 (55%)
	HIGH	4 (2%)	3/3 (100%)	1/4 (25%)	0/4 (0%)
			NS	0.035	0.095
GROUP 2	SOKAL	N	CCyR	MMR	CMR
Imatinib 400 +high dose imatinib MEDIAN FOLLOW UP 56,8 months N=27	LOW	11 (41%)	9/11 (82%)	9/10 (90%)	5/10 (50%)
	INTER	9 (33%)	7/8 (87%)	6/8 (75%)	4/8 (50%)
	HIGH	7 (26%)	6/7 (86%)	6/7 (86%)	2/7 (28%)
			NS	NS	NS
	HASFORD				
	LOW	13 (48%)	9/12 (75%)	9/12 (75%)	5/12 (42%)
	INTER	10 (37%)	10/10 (100%)	9/9 (100%)	6/9 (67%)
	HIGH	4 (15%)	3/4 (75%)	3/4 (75%)	0/4 (0%)
			NS	NS	0.08
GROUP 3	SOKAL	N	CCyR	MMR	CMR
Imatinib 400 +high dose imatinib + 2ºTKI MEDIAN FOLLOW UP 40,3 months N=9	LOW	1 (11%)	1/1 (100%)	0/1 (0%)	0/1 (0%)
	INTER	6 (67%)	2/6 (33%)	1/6 (17%)	0/6 (0%)
	HIGH	2 (22%)	1/2 (50%)	0/2 (0%)	0/2 (0%)
			NS	NS	NS
	HASFORD				
	LOW	2 (22%)	1/2 (50%)	0/2 (0%)	0/2 (0%)
	INTER	5 (56%)	3/5 (60%)	1/5 (20%)	0/5 (0%)
	HIGH	2 (22%)	0/2 (0%)	0/2 (0%)	0/2 (0%)
			NS	NS	NS
GROUP 4	SOKAL	N	CCyR	MMR	CMR
High dose imatinib MEDIAN FOLLOW UP 33,3 months N=11	LOW	6 (54%)	4/5 (80%)	5/6 (83%)	4/6 (67%)
	INTER	2 (18%)	2/2 (100%)	2/2 (100%)	2/2 (100%)
	HIGH	3 (27%)	1/2 (50%)	2/3 (67%)	2/3 (66%)
			NS	NS	NS
	HASFORD				
	LOW	5 (46%)	4/4 (100%)	5/5 (100%)	4/5 (80%)
	INTER	4 (36%)	2/3 (67%)	3/4 (75%)	3/4(75%)
	HIGH	2 (18%)	1/2 (50%)	1/2 (50%)	1/2 (50%)
			NS	NS	NS
GROUP 5	SOKAL	N	CCyR	MMR	CMR
Imatinib 800 + 2ºTKI MEDIAN FOLLOW UP 31,3 months N=17	LOW	8 (50%)	4/8 (50%)	5/8 (63%)	1/8 (13%)
	INTER	5 (31%)	2/2 (100%)	3/5 (60%)	1/5 (20%)
	HIGH	3 (19%)	0/3 (0%)	0/2 (0%)	0/2(0%)
			0.084	NS	NS
	HASFORD				
	LOW	3 (19%)	2/3 (67%)	1/3 (33%)	1/3 (33%)
	INTER	10 (62%)	3/7 (43%)	6/10 (60%)	1/10 (10%)
	HIGH	3 (19%)	1/3 (33%)	1/2 (50%)	0/2(0%)
			NS	NS	NS







Follow-up of the different groups: Complete cytogenetic response: with regards to the best response, the CCyR rate was lower in patients with Im400-HDIm-2GTKI (44%) and patients with Im 400-2GTKI (50%). In the other groups, the rate was 80% (Im400) 1), 84.6% (Im400-HDIm) and 78% (HDIm) Pearson Chi-Square 12,867(a); P=0,012. The CCyR cumulative incidence was also lower in patients with Im400-HDIm-2GTKI and Im 400-2GTKI in comparison with the other groups, although second line response was faster in patients who changed to 2GTKI after Im400. Major molecular response: concerning the best response, the MMR rate was lower in patients with Im400-HDIm-2GTKI (0%) and patients with Im 400-2GTKI (19%). In the other groups, the rate was 52% (Im400) 1), 56% (Im400-HDIm) and 73% (HDIm) Pearson Chi-Square 17,405(a)P=0,002. The MMR cumulative incidence was higher in the HDIm group, lower in those treated with Im400-HDIm-2GTKI, and intermediate and similar in the other three groups. Complete molecular response: regarding best response, the CMR rate was lower in patients with Im400-HDIm-2GTKI (0%) and patients with Im400-2GTKI (23%). In the other groups, the rate was 48% (Im400) 1), 44% (Im400-HDIm) and 72% (HDIm) Pearson Chi-Square 17,405(a) P=0,002. The CMR cumulative incidence was higher in the HDIm group, and nil in those treated with Im400-HDIm-2GTKI. *Conclusions.* A High response rate (genetic and molecular) was seen in the majority of patient treated with imatinib. Patients treated with upfront HD appeared to have a faster molecular response. High-risk Hasford patients showed a worse outcome, independently of treatment chosen.

Aggressive B-cell lymphomas

0665

WITHDRAWN BY AUTHOR

0666

LONG-TERM OUTCOME OF 309 YOUNG PATIENTS WITH UNTREATED DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL) AT POOR PROGNOSIS: A POOLED ANALYSIS FROM GIMURELL AND INTERGRUPPO ITALIANO LINFOMI (IIL)

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Background. DLBCL patients at poor prognosis had a dismal prognosis; investigational approaches in the context of clinical trials were experienced. *Aims.* A pooled analysis was conducted to test the role of high-dose chemotherapy (HDC) and autologous stem-cell transplant (ASCT), Rituximab, dose-dense MegaCEOP *vs.* third generation MACOP-B chemotherapy and involved-field radiotherapy (IF-RT) in first line treatment in young DLBCL at poor prognosis. Patients and *Methods.* 309 untreated patients <61 years were enrolled from 1986 to 2006 into four consecutive Italian multicenter trials; 42 into a phase II study and treated with MACOP-B chemotherapy (Vitolo U, J Clin Oncol 1992); 40 into a phase II trial with 8 weekly MACOP-B as induction therapy, followed by HDC with 2 courses of MAD and BEAM and ASCT (Vitolo U, J Clin Oncol 1997); 107 into a phase III randomized trial, 48 treated with high dose sequential (HDS) chemotherapy with ASCT and 59 with 6 be-weekly infusions of MegaCEOP, respectively (Vitolo U, Haematol 2005); 120 into a phase II trial with an induction phase with dose-dense chemoimmunotherapy Rituximab-MegaCEOP for 4 cycles followed by HDC and ASCT (Vitolo U, Haematol 2009). IF-RT was performed at the end of treatment as consolidation of bulky disease or residual disease. A Cox proportional hazard model was performed for Overall Survival (OS) and Progression-Free Survival (PFS) to estimate the Hazard Risks (HR) of the four treatment variables (with/without Rituximab, HDC+ASCT, new regimens *vs.* third generation regimen MACOP-B, radiotherapy yes/no), adjusted by age adjusted-International Prognostic Index (aa-IPI), age and sex.

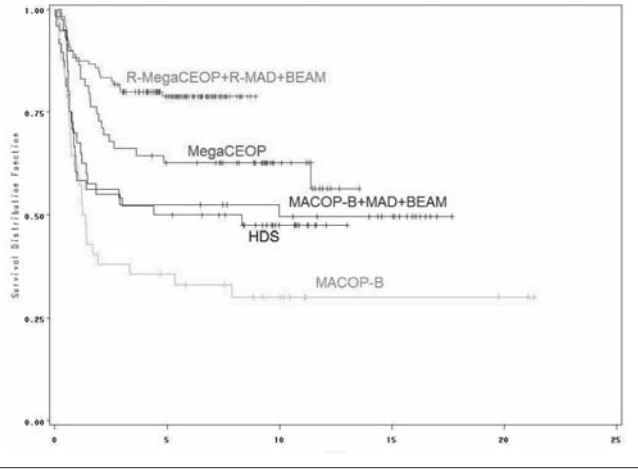

Figure 1.

Results. Clinical characteristics were: median age 44 (15-60) years; Performance Status >2 59%, 31% had BM involvement, 53% bulky disease, 84% LDH >normal, 12/19/69% stage II/III/IV respectively; according to aa-IPI 51% were at Intermediate-High and 39% at High risk. Rituximab was performed in 120 patients; IF-RT in 108; new generation regimens in 227 and MACOP-B in 82. As intention to treat, ASCT was scheduled for 208 patients; 171 patients (82%) did it, 39 did not because of: progression disease in 22, toxicity in 9 and poor mobilization in 6. Response rate in all 309 patients was: complete response 69%, partial 7%, no response 18% and 6% toxic deaths. With a median follow-up of 10 years, 10-year OS and 10-year PFS were: 59% (95%CI: 53-65) and 48% (95%CI: 41-55). OS for schemes was reported in Figure 1. Secondary haematological malignancies or solid tumour were observed in only three patients. The Cox's multivariable model showed an improvement of the outcome with the use of Rituximab (HR=0.36, 95% CI=0.21-0.63, P .0003) and IF-RT (HR=0.42, 95% CI: 0.27-0.66, P.0002), while no clear benefit was represented by new regimens (HR=0.86, 95%CI: 0.57-1.31, P.482) and HDC (HR=0.94, 95%CI: 0.62-1.41, P .751). *Conclusions.* with the limit of a retrospective analysis, the use of Rituximab and the IF-RT seems to represent an important role in the treatment of young patients with untreated DLBCL at poor prognosis. Randomized trials are ongoing to evaluate the real impact of HDC+ASCT supplemented with Rituximab compared to standard or dose-dense chemoimmunotherapy.

0667

CLINICAL FINDINGS AND TREATMENT OF ELDERLY PATIENTS WITH DIFFUSE LARGE B-CELL LYMPHOMA: THE ANALYSIS OF THE 1425 PATIENTS INCLUDED IN CZECH LYMPHOMA PROJECT (CLP)

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Background. Diffuse large B-cell lymphoma (DLBCL) is most frequent subtype of NHL in many European countries. Large trials on elderly DLBCL patients are infrequent and if, predominantly the fit patients participate in these trials. We have initiated Czech Lymphoma Project to describe the clinical and laboratory findings in unselected cohort of patients. *Aim.* To evaluate the treatment modalities, clinical outcome and prognostic factors in elderly patients with DLBCL. *Methods.* DLBCL forms 43% of newly diagnosed NHL per year in the Czech Republic. The median age is 63 years and 59% of patients are 60 years old or older. We analyzed data of 1,425 patients with confirmed DLBCL older than 59 years and diagnosed before 31/12/2008. Those with CNS involvement were excluded. Histology was reviewed in 92.7% of cases. The median age at diagnosis was 70 years (60-94); Ann Arbor stages were as follows: I (21.4%), II (26.5%), III (16.7%), IV (35.4%). Extranodal disease was present in 66.3% of cases. IPI and age-adjusted IPI (aaIPI) scores were: low (L) 23% and 22%, low-intermediate (LI) 26% and 30%, intermediate-high (IH) 25% and 27%, high (H) 26% and 21%, respectively. First-line treatment was initiated in 86.7%. The most frequent treatment modality was an anthracycline-containing (CHOP) regimen (78%) with a median of 6 cycles. An intensive chemotherapy was administered to only 2.3% of patients. Rituximab was added to chemotherapy in 58% (a median of 6 doses) and radiotherapy in 25% of patients. The treatment response was assessable in 1,076 (87.1%) of the treated patients. PET scan was used in 139 cases.

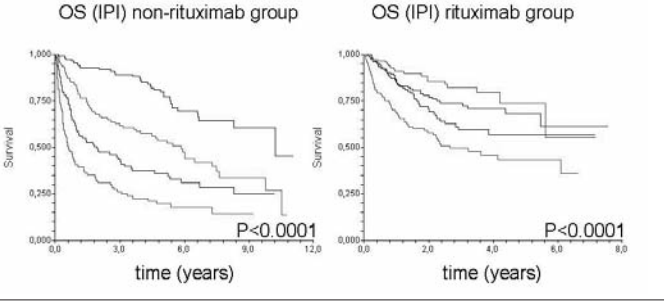

Figure 1.

Results. Generally, complete remission (CR), unconfirmed CR, partial remission and stable disease were achieved in 66.3%, 9.9%, 12.5% and 2.5%, respectively. Only 8.8% of patients progressed on therapy. The ORR reached 88.7%. PET-negative CR was achieved in 114 (82%) of 139 assessable cases. After a median follow-up of 2.7 years, 574 patients (40.3%) are alive without relapse, 313 (22%) relapsed or progressed and 517 (36.3%) died. The overall survival at 3 years (3y OS) reached 59.6% (95% CI 0.57-0.63); the event-free survival at 3 years (3y EFS) was 50% (95% CI 0.47-0.53). The 3y OS stratified by IPI and aaIPI: (L) 85%/ 84%, (LI) 67%/67%, (IH) 50%/50%, (H) 37%/32%. The 3y EFS according to IPI and aaIPI: (L) 76%/76%, (LI) 58%/57%, (IH) 39%/39%, (H) 28%/24%. Rituximab improved both 3y OS (55% *vs.* 65%, P<0.0001) and 3y EFS (44% *vs.* 59%, P<0.0001). OS improvement was seen in all risk groups except low-risk patients. PET negativity after treatment predicts longer OS and EFS (P=0.0011 and P=0.0005, respectively). Multivariate analysis identified nonCR status, age≥70, PS≥2 (ECOG), LDH and rituximab application as independent predictors of OS. Similarly, nonCR status, age≥70, IH/H aaIPI score, LDH and rituximab application independently predict EFS. Summary. Current treatment modalities lead to long-term remission in 2/3 of elderly DLBCL patients. Survival of high-risk patients remains poor. Rituximab significantly reduces the risk of death and disease progression especially in IH/H IPI subgroups. PET negativity is an essential prerequisite for long-term CR. Acknowledgements. IGA NR/9502-3.

0668

LYMPHOCYTOPENIA MAY ADD TO THE PREDICTION ACHIEVED BY THE REVISED INTERNATIONAL PROGNOSTIC INDEX (R-IPI) IN PATIENTS WITH DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL) TREATED WITH RITUXIMAB-CHOP (R-CHOP)

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Introduction. Low absolute lymphocyte counts (ALC), generally <1.0×10^9/L, have been recently considered as an adverse prognostic factor in DLBCL, both prior and after the introduction of Rituximab. However, published experience in the Rituximab era is limited to very few moderately sized series (approximately 100 patients each). Therefore, the prognostic significance of lymphocytopenia in patients with DLBCL treated with R-CHOP deserves further investigation. *Aim.* To determine the frequency of lymphocytopenia and to evaluate its correlation with other baseline features and the outcome of patients with DLBCL treated with R-CHOP. A predefined cutoff of 0.84×10^9/L was used to define severe lymphocytopenia, as proposed by Cox MC *et al.* (Leuk Lymphoma 2008; 49: 1745-51). Patients and *Methods.* Among 482 patients with DLBCL, who were treated with R-CHOP or similar anthracycline-based combinations in the participating centers, the ALC was available in 399. Gender and the 5 individual components of the IPI, as well as the R-IPI, were recorded at baseline, and failure free survival (FFS) (including early toxic deaths as events) and overall survival (OS) were determined as endpoints for the outcome. *Results.* The median age of the patients was 65 years (18-91), and 57% were males. According to the R-IPI, 20% of patients were classified as Very Good Risk (VG), 47% as Good Risk (G), and 33% as Poor Risk (P). The median ALC was 1.504×10^9/L; 21% of the patients had an ALC <1.0×10^9/L and 14% had severe lymphocytopenia according to the predefined cutoff of 0.84×10^9/L. The ALC was significantly correlated with advanced stage, elevated LDH, age >60 yrs and higher R-IPI, but not with poor performance status (marginal association), multiple extranodal involvement or gender. Severe lymphocytopenia was associated with inferior 3-yr FFS (79% *vs.* 67%, P=0.002) and 3-yr OS (83% *vs.* 73%, P=0.005). The R-IPI was also highly discriminative for both FFS and OS. In multivariate analysis, severe lymphocytopenia added independent prognostic information to R-IPI for both FFS and OS with hazard ratios in the order of 2.0. The presence of severe lymphocytopenia identified an even worse subgroup including19% of the poor risk R-IPI patients with 3-yr FFS and OS of 38% and 49% *vs.* 57% and 62% for the remaining poor risk R-IPI patients without severe lymphocytopenia. On the contrary, severe lymphocytopenia had not any prognostic impact in Very Good and Good risk patients according to the R-IPI. *Conclusions.* A severe depression of ALC (<0.84×10^9/L) was present in 14% of DLBCL patients, being associated with several other adverse prognostic factors. The presence of severe lymphocytopenia provides prognostic information independent from that obtained by the R-IPI and classifies patients with Poor risk R-IPI into two subgroups with different outcomes. Our results reinforce the view that lymphocytopenia might be incorporated in prognostic models for DLBCL in the R-CHOP era.

0669

PROGNOSTIC SIGNIFICANCE OF INTERIM 18F-FDG PET/CT FOR TREATMENT OF DIFFUSE LARGE B CELL LYMPHOMA IN POST-RITUXIMAB ERA

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FDG-PET/CT, a functional imaging modality used for staging and monitoring response to treatment of malignant lymphoma, has a higher sensitivity and specificity than conventional imaging. We prospectively investigated that PET/CT may provide additional prognostic information in interim response assessment prior to completion of chemotherapy in diffuse large B cell lymphoma (DLBCL). Patients and *Method.* 153 newly diagnosed patients with diffuse large B cell lymphoma were enrolled from Aug. 2004 to July 2009 in single institution. PET/CT analysis was performed at the time of diagnosis and after the 3rd or 4th R-CHOP chemotherapy. The clinical stage and response of the patients were assessed according to revised response criteria for aggressive lymphomas (Cheson, J Clin Oncol, 2007). The limited-stage patients were treated with four cycles of R-CHOP chemotherapy and followed by involved field radiation therapy (IFRT). The advanced-stage (III/IV) patients were treated with eight cycles of chemotherapy. However, the advanced-stage patients who were older than sixty-five, if they had a complete response (CR) by interim analysis were treated with only six cycles. *Results.* Median age was 60 years (range: 17 - 85). Sixty-four patients (41.8%) presented in advanced stages and 26 (17.4%) had bulky mass (≥10 cm). The International Prognostic Index was 47.1% of low risk, 19.6% of low-intermediate, 17.6% of intermediate-high and 15.7% of high risk. 110 patients (71.9%) achieved CR, 37 patients (24.2%) achieved PR, 4 patients (2.6%) showed stable or progressive disease and 2 patients (1.3%) was non-measurable by interim revised IWC. 111 patients (72.5%) had a negative, while 40 (26.1%) remained positive by interim PET/CT. Five patients (3.3%) revealed false positive-FDG and one false negative-FDG by tissue biopsy. After following median 30.8 months, 3-year overall (OS) and progression free survival (PFS) rate was 74.2±3.9% and 71.0±3.9%, respectively. The relapse rate was significantly different between FDG-positive (60.0%) and FDG-negative (12.6%) (P=0.000). Figures showed the Kaplan-Meier estimates of OS and PFS depending on the interim PET/CT. *Conclusions.* Interim PET/CT analysis was a significantly predictive value of disease progression and survival of DLBCL after R-CHP chemotherapy. The patients with PR or positive according to interim IWC and PET/CT analysis should be considered for an intensive therapeutic plan.

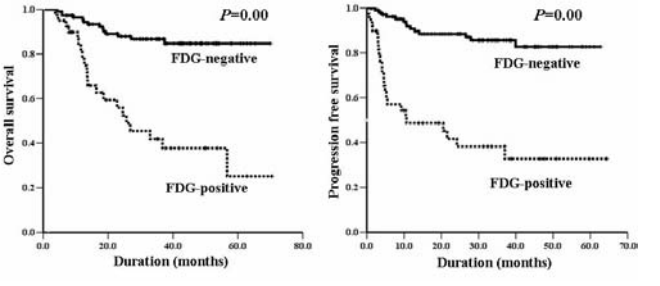

Figure 1.

0670

IMPROVEMENT OF OUTCOME IN DIFFUSE LARGE B-CELL LYMPHOMA AFTER INTRODUCTION OF RITUXIMAB IN COMBINATION WITH CHEMOTHERAPY. EVALUATION IN DAILY PRACTICE IN A COHORT OF 410 PATIENTS TREATED AT A SINGLE INSTITUTION BETWEEN 1996 AND 2008

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Background. Rituximab (R) in combination with chemotherapy (CT) has dramatically improved the prognosis of patients with Diffuse Large B-Cell Lymphoma (DLBCL). Good results have been observed in clinical trials or in the context of curative care in population-based analyses. *Aims.* Whether this therapeutic effect is observed in daily practice without any selection for patient age, type of associated CT or curative/palliative intent at diagnosis needs to be controlled. We proposed to perform this analysis in an unselected population of DLBCL patients. *Methods.* From 1996 to 2008, 410 patients with DLBCL received first-line therapy at our institution. The median age of the patients was 64 years (range, 19-96). Treatment consisted of CHOP-like CT (218 patients, 53%), high-dose CHOP CT (129 patients, 31%), other CT with anthracyclin (49 patients, 12%) or without anthracyclin (10 patients, 3%), radiotherapy and surgery in 4 patients (1%). Rituximab was associated to CT for 267 patients: respectively 17 and 250 before and after approval of rituximab for DLBCL in March 2002. *Results.* With a median follow-up of 60.2 months (95%CI [52.2-62.5]), the 5-year overall survival (OS) of the cohort is 67% (CI95% [62-72]). At last evaluation, 27 patients (7%) had died of toxicity after first-line therapy, 81 of progressive disease (20%) and 24 of other causes (6%). Amont these last patients, eight died of solid neoplasia and one of myelodysplasia. The 5-year Event-Free Survival (EFS) of the cohort, calculated from the date of diagnosis to the first occurrence of events (relapse, disease progression, or death of any cause), was 61% (CI95% [56-66]). Comparison of OS between patients treated with or without R showed that patients receiving R had a better outcome, with a 5-year OS of 72% (CI95% [66 - 78]) compared to 59% (CI95% [51 -67]) for patients not treated with R (P<0.001). EFS was also improved with a 5-year EFS of respectively 66% (CI95% [59 - 72]) and 53 % (CI95% [45 - 61]) (P<0.01) for patients treated with and without R combined to CT. Evaluation by age groups showed that this effect was observed for patients older than 60: 5-year OS was 65% (CI95% [56 - 74]) and 41% (CI95% [31 - 53]) (P<0.01) for patients treated and not treated with CT combined with R, respectively. In our cohort, the influence of R was not observed for patients younger than 60: 5-year OS and EFS were 82% (CI95% [70 - 89]) and 75% (CI95% [63 - 84]) for patients treated without R and 81% (CI95% [72 - 88]) and 77% (CI95% [68-84]) for patients with R (P=0.99 for OS; P=0.79 for EFS). *Conclusions.* This study confirmed the improvement of DLBCL prognosis by R in a context of daily practice without the selection bias of clinical trials. However this effect was observed for elderly patients but not for younger patients who were treated in our institution mainly by dose-dense chemotherapy (70%). The prognostic impact of R will be also presented by age-adjusted IPI, type of CT and treatment response.

0671

SERUM SORUBLE CD27 LEVEL DETERMINES CLINICAL OUTCOME IN PATIENTS WITH DIFFUSE LARGE B-CELL LYMPHOMA IN RITUXIMAB ERA

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Purpose. Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous entity, with patients exhibiting a wide range of outcomes. The introduction of rituximab to CHOP (R-CHOP) has significantly altered improvement in survival. This raises concern regarding the utility of previously identified prognostic factors. Before rituximub era, some investigators have suggested that serum levels of some cytokines and their soluble receptors might reflect tumor growth and host tumor responses. CD27 belongs to tumor necrosis factor - receptor family, especially express on lymphcytes, and CD27 expression of variable intensity was detected on almost all immature and mature malignant B cells, and also the highest levels of serum soluble CD27(sCD27) were reported in chronic lymphocystic leukemia and low-grade non-Hodgkin's lymphoma. We have previously assessed that sCD27 was strong prognostic factor in patients with DLBCL who received CHOP without rituximab . The aim of the present study is to re-assess the prognostic significance of serum sCD27 in DLBCL treated with rituximab. In addtion we assessed sCD27 with subtype DLBCL, GCB type and non-GCB type, and we analyzed CD27 stain of tumor tissue in immunohistochemistry at the diagnosis. Materials and *Methods.* Consecutive 143 previously untreated patients with DLBCL prospectively participated in this study between 2002 and 2008. The patients were treated with 6-8 cycles of R-CHOP. Serum sCD27 was determined by ELISA, and we classified subgroups of DLBCL according to Hans *et al.*, and additionally we analyzed CD27 stain of tumor tissues. *Results.* In all patients with DLBCL, the median of serum sCD27 level was 157.61 ng/mL (range 1.6 - 1488 ng/mL). Various poor prognostic features, such as poor PS, many extranodal sites, advanced disease (CS III/IV), increased LDH and elderly people were strongly associated with high serum CD27 levels. The median serum sCD27 levels of the different IPI risk groups were as follows: 42.6 ng/mL for the L risk; 106 ng/mL for the LI risk; 218.1 ng/mL for the HI risk; 405.2 ng/mL for the H risk, respectively (P<0.0001). A similar result was provided in rivised IPI (42.6 ng/mL for the very good risk; 68.8 ng/mL for the good risk; 326.5 ng/mL for the poor risk, respectively P<0.0001). In addition, the serum sCD27 correlated with LDH, CRP (LDH: r=0.47, P<0.0001; CRP r=0.58, P<0.0001). Patients with high sCD27 (200 ng/mL and over) at onset had significantly lower overall survival rates (4-year: 54.4%), than those with low sCD27 (4-year: 73.5 %), respectively (P<0.0005). and there were no difference with median serum sCD27 levels in GCB and non-GCB subgroups, but CD27 positive cells were observed in GCB types more than in non-GCB types (GCB: 30%, non GCB: 6.78%; P=0.0007), and patients with CD27 positive tumor in GCB type had poorer prognoses than those of CD27 negative. *Conclusion.* These results suggest that, in even rituximab era, a high serum CD27 level predicts a poor prognosis of DLBCL and may be a useful biomarker for selecting appropriate treatment. Upfront high dose chemotherapy might be well indicated for DLBCL patients with high serum sCD27.

0672

CYTOGENETIC ANALYSIS OF BONE MARROW WITH INVOLVEMENT OF DIFFUSE LARGE B-CELL LYMPHOMA IN KOREA

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Background. Bone marrow (BM) evaluation is essential in routine staging of diffuse large B-cell lymphoma (DLBCL), because bone marrow involvement (BMI) affect both prognosis and treatment strategies. Cytogenetic studies have been used increasingly with the expending knowledge on genetic characteristics of DLBCL, however, little information is available on the clinical utility of cytogenetic studies in BM specimens for detection of BMI and cytogenetic characteristics of DLBCL with BMI and its clinical impact. *Aims.* The aim of this study was to assess the diagnostic utility of cytogenetic studies in comparison with morphologic findings in BM specimens from DLBCL patients and to characterize features and to determine the prognostic impact of chromosomal abnormalities in DLBCL with BMI. *Methods.* We analyzed results of histologic findings and karyotypes using the conventional G-banding technique on BM aspirates obtained for staging or follow-up purpose from 503 patients who diagnosed DLBCL from 1996 to 2009 in Seoul National University Hospital. In 148 patients, interphase fluorescent *in situ* hybridization (FISH) were also performed using commercially available probes including an IGH dual color break-apart rearrangement probe, a p16/CEP 9 dual color probe, and a BCL6 dual color break-apart rearrangement probe (all from Vysis). *Results.* 137 (27.2%) cases showed BMI of DLBCL cells by histological examination. Chromosomal aberrations were detected in 90 (17.9%) cases among 503 patients and 68/137 (49.6%) cases with BMI showed abnormal karyotypes. Meanwhile, 22/366 cases (6.0%) with no evidence of BMI by histologic examination showed abnormal karyotypes. Of 148 patients for whom FISH results were available, 44.0% (33/75) of cases with histologic BMI showed abnormal FISH results, while 1 patient among 73 patients with no evidence of BMI showed abnormal FISH *Results.* When compared with G-banding, 13/34 (38.2%) cases with abnormal FISH results showed normal karyotypes by G-banding. Among 90 patients with abnormal karyotypes with G-banding, complex karyotypes were observed in 59 cases (65.6%), hyperdiploidy in 15 cases (16.7%) and translocations in 46 patients (51.1%). Abnormalities affected chromo-

somes 1 (43.3%, 39/90), followed by Chromosome 3 (35.6%, 32/90), Chromosome 14 (34.4%, 31/90), Chromosome 6 (28.9%, 26/90), and Chromosome 18 (28.9%, 26/90) in descending order of frequency. The most commonly observed specific abnormalities were: rearrangements involving 14q32 (23.3%), 8q24 (13.3%), 19q13 (13.3%), 3q27 (11.1%), 11q23 (11.1%), 1p25-p36 (8.9%), 18q21-q23 (8.9%), and 19p13 (8.9%); deletions of 6q12-q25 (14.4%), 1p21-p32 (12.2%), and 7q22-q34 (8.9%); duplications of 1q21q42 (8.9%); chromosomal gain of 18 (14.4%), 7 (11.1%), and 3 (8.9%); chromosomal loss of 13 (8.9%). The prognostic analysis of patients with BMI showed that patients with complex karyotypes were significantly decreased overall survival than patients with normal karyotypes (9.0 *vs.* 51.1 months, P<0.001). However, 17 patients with minor chromosomal abnormalities with G-banding did not affect survival (P=0.575). *Conclusions.* In addition to histologic examination, conventional cytogenetic study with G-banding technique and FISH analysis could be useful tools in detecting BMI. Complex karyotype was an adverse prognostic factor, and further analysis of larger series of patients with DLBCL with BMI may clarify the prognostic stratification by cytogenetic results.

Table 1. Frequent chromosomal abnormalities in 90 patients with abnormal karyotypes.

Chromosome	No. of abnormal cases	Rearrangement (no. cases)	Deletion (no. cases)	Duplication (no. cases)	Gain (no. cases)	Loss (no. cases)
X	16				+X (5)	−X (4)
Y	14					−Y (14)
1	39	1p36 (8), 1q24 (6)	1p21-p32 (11)	1q21q42 (8)		
2	24		2p21-p23 (4), 2q31-q35 (6)			−2 (5)
3	32	3q27 (10)	3p13-p25 (5), 3q21-q25 (4)		+3 (7)	
4	13		4q31 (4)			−4 (5)
5	15		5q13-q35 (5)		+5 (4)	−5 (4)
6	26	6p23-p25 (4)	6q12-q25 (13)			−6 (4)
7	23		7q22-q34 (8)		+7 (10)	
8	21	8q24 (12)				−8 (4)
9	21	9p24 (6)	9q10-q22 (5)			
10	15					−10 (4)
11	23	11q25 (10)	11q13-q25 (5)			
12	17	12q24 (4)	12p11-p13 (4)			
13	22	13q10-q12 (4), 13q32-q34 (5)	13q21-q34 (4)			−13 (8)
14	31	14q32 (21)				−14 (4)
15	19	15p10-p11 (4)				−15 (5)
16	11	16q22-q24 (6)				
17	18	17p12 (4), 17q23 (4)	17p11-p12 (4)			−17 (4)
18	26	18q21-q23 (8)			+18 (13)	
19	22	19p13 (8), 19q13 (12)				−19 (4)
20	6					
21	13				+21 (4)	
22	13					−22 (6)

0673

USE OF STATINS AND PROGNOSIS IN PATIENTS WITH CD20-POSITIVE 'DE NOVO' DIFFUSE LARGE B CELL LYMPHOMA (DLBCL) TREATED WITH RITUXIMAB CONTAINING REGIMENS

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Background. Statins may interfere with anticancer treatment by different promoting or inhibiting mechanisms. A recent study reported a better event-free survival in follicular lymphoma patients treated with statins as compared to non-users. So far, no data on the possible prognostic impact of statin use in '*de novo*' DLBCL have been reported. There is some evidence that statins may reduce the effect of Rituximab treatment by changing the expression of CD20 molecules on the surface of B-cells. However, so far, no studies have examined whether this may have a impact on treatment outcome in CD20 positive DLBCL patients using statins. *Aims.* To investigate whether the use of statins was associated with the clinical outcome of patients with CD20-positive '*de novo*' DLBCL treated with Rituximab containing regimens. *Methods.* We constructed a historical cohort based on the population-based Danish lymphoma registry LYFO and included patients with CD20-positive '*de novo*' DLBCL treated with Rituximab containing regimens diagnosed before 01.01.2008 at the haematological departments of Aarhus and Aalborg . Demographic and clinico-pathological characteristics were obtained from the LYFO database. Data on the use of statins, comorbidities and death were obtained from the prescription databases of the Central Denmark Region and the North Denmark Region, the Danish National Registry of Patients and the Danish Civil Registration System. We used regression analysis to compute adjusted mortality and event ratios within 30 months from diagnosis. The analysis was adjusted for age, IPI and comorbidity. *Results.* A total of 280 patients were analysed. Of these, 11 (3.9%) were excluded due to missing clinical data. Of the remaining 269 patients, 39 (14.5%) were statin users. Overall survival (OS) and event-free survival (EFS) proportions at 30 months after diagnosis were similar between statins users and nonusers (OS: 32.5% *vs.* 29.2% and EFS: 35% *vs.* 34.6%, respectively). Age and comorbidity showed a significant difference between the two groups. In the statin-treated group 12,5% of the patients were under 60 years of age (mean age 70 years) and 70% had a history of comorbidity, whereas corresponding values among non-users were 35% (mean age 64.3 years) and 41.3%, (P<0.01 and P<0.001) respectively . As a result of this the adjusted mortality ratios for OS and EFS comparing statin users with nonusers were: 0.76 (0.38-1.53) and 0.69 (0.35-1.37), respectively. *Conclusions.* Our data do not support the hypothesis, that statin use inhibits Rituximab activity. Further studies are warranted to investigate whether the use of statins may have impact on treatment outcome in selected subsets of patients with DLBCL and when evaluated over a longer follow-up period.

0674

TOLERANCE OF R-CHOP-21 FOR DIFFUSE LARGE B-CELL LYMPHOMA IN AN UNSELECTED POPULATION AND IMPACT OF DOSE REDUCTION ON OUTCOME

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Background. 6-8 cycles of R-CHOP-21 is widely regarded as standard initial therapy for advanced diffuse large cell lymphoma (DLCL) with studies demonstrating high response rates and durable remissions even in those over sixty. However, there is very little published data on R-CHOP-21 tolerance in unselected patients or the impact of dose reduction on outcome. *Aims.* To establish the proportion of unselected patients in routine practice deemed able to receive full dose R-CHOP-21 for advanced DLCL and the impact of dose reduction on response rates, response duration and survival. *Methods.* All patients with stage 3 or 4 *de novo* DLCL (including follicular lymphoma grade 3b) commencing therapy between 1st Sept 2005 and 31st November 2009 were identified by computerised records searches at three centres with similar patient populations and supportive care protocols. Patients with known pre-existing low grade lymphoma, prior chemo- or radiotherapy for a haematological disorder or HIV were excluded. Patient records were reviewed for relevant data and staging and response re-determined. Overall and progression free survival were calculated from date of first chemotherapy.

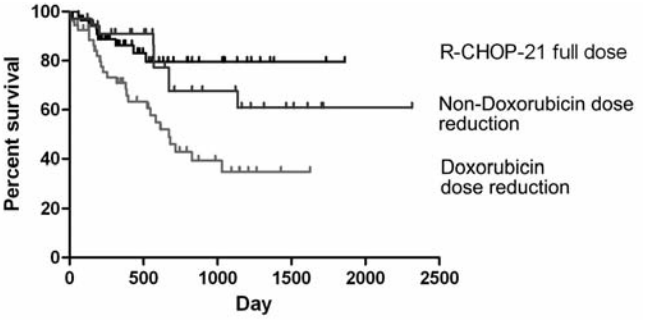

Figure 1. Overall Survival Curves for R-CHOP-21.

Results. 170 patients were identified. 14 received R-CHOP-14, 2 received other regimens, records were unavailable for 1, all these were excluded from further analysis. Of the remaining 153, 60 completed 6 cycles of full dose R-CHOP-21 (Rituximab 375 mg/m^2, Cyclophos-

phamide 750 mg/m^2, Vincristine 1.4 mg/m^2, maximum dose 2 mg, and prednisolone 100mg for 5 days) and 90 had some dose and/ or cycle number reduction. Treatment cessation for death or progressive disease during full dose therapy (3 patients) was not considered dose reduction. Doxorubicin dose was reduced in 54 (median 33% of full 6 cycle dose), cyclophosphamide in 37 (median 80% full dose), vincristine in 59 (median 50% full dose), Rituximab in 7 (range 50-83% full dose). 15 had ≤5 cycles with reduced dose per cycle of at least one agent (median 4 cycles). The commonest reasons for dose reduction were age (55%), neuropathy (14%) and poor tolerance (14%). There was no difference in IPI (median 3) or ECOG performance status (median 1) between those receiving full dose or not. Median age was higher in dose and/ or cycle reduction patients than full dose (78 *vs.* 65 years). Median IPI, performance status and age did not differ between doxorubicin dose reduction patients and those receiving only other drug reductions. Median follow up was 21 months. Overall survival, progression free survival and response rates were determined for full dose and reduction of each drug. Reduction in ≥1 of vincristine, cyclophosphamide, rituximab did not produce significant reduction in response rates or survival. Reduction in doxorubicin produced lower median overall survival (21 months *vs.* not reached, P=0.0022, Figure1) and overall response rates (97% *vs.* 82%, P=0.0025, Fisher's exact test). Summary/ Conclusions. Outside clinical trials, a substantial proportion (59%) of patients with advanced DLCL are deemed unfit to receive R-CHOP-21 at full dose. Reduction in agents other than doxorubicin has no significant impact on response rates or overall survival and, where dose reduction is necessary, is preferable to doxorubicin attenuation.

0675

SINGLE NUCLEOTIDE POLYMORPHISMS IN FCGRIIA AND FCGRIIIA MAY AFFECT RESPONSE RATE BUT NOT SURVIVAL IN NEWLY DIAGNOSED DLBCL PATIENTS TREATED WITH RITUXIMAB AND CHOP CHEMOTHERAPY

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Background. Single nucleotide polymorphisms (SNPs) of Fcγ receptors (FcγRs): FcγIIA and FcγRIIIa in effector cells may predict response rate and clinical outcome in B-cell lymphoma patients (pts) treated with immunochemotherapy. However, reports on the prevalence of FCGR2A and FCGR3A genes SNPs and their influence on overall survival (OS) and progression free survival (PFS) in pts with diffuse large B-cell lymphoma (DLBCL) are inconsistent. *Aims.* To evaluate the frequency of FCGR2A-131H/R and FCGR3A-158V/F polymorphisms in DLBCL pts treated with R-CHOP and possible relation between SNPs and characteristics of pts, response rate, frequency of neutropenia, OS and PFS. *Methods.* Newly diagnosed DLBCL pts subsequently treated with R-CHOP between 2004 and 2007 at our institution were studied retrospectively. Peripheral blood was taken during R-CHOP therapy. Genotyping was performed on DNA obtained from peripheral blood using TaqMan SNP Genotyping Assays (Appliedbiosystems, USA) and confirmed by sequencing. Response to treatment and endpoints were determined by 1999 Cheson criteria. Statistics was done with SPSS 12.0. *Results.* Patient characteristics: n=87, median age (range): 57 (25-81), male/female: 41/46, Ann Arbor stage III-IV: 74%, LDH>normal: 48%, E sites: 59%, IPI score 0-2: 76% of pts. Median (range) number of R-CHOP cycles was 6 (4-8). During a median observation time of 40 months, 21 pts (44%) had disease progression with a median time to progression of 9 months. There were 17 deaths. SNP distribution was: H/H-31%, H/R-46%, and R/R-23% for FCGR2A, and V/V-17%, V/F-40%, and F/F-43% for FCGR3A. There were no significant differences in SNPs distribution by age, sex, Ann Arbor stage, LDH level, IPI, or primary site of DLBCL. A higher CR rate was observed in FCGR2A H/H (74%) and H/R (75%) alleles compared with R/R (55%; P=0.046). FCGR3A V/V allele was associated with more than 7-fold higher probability of CR (P=0.046, OR 7.492, 95%CI 1.039-54.028). Other predictive factors for CR were: ECOG PS - 0 (P=0, OR 18,705, 95%CI 4.369-80.06), IPI <3 (P=0.03 OR 6.587, 95%CI 1.206-35.993), and age>60 (P=0.014, OR 10.076, 95%CI 1.59-63.835). Neither FCGR2A nor FCGR3A allele were significantly related to OS and PFS. Normal LDH level was predictive for longer OS (P=0.021 HR 0.338, 95%CI 0.134-0.852), and IPI score<3 - for longer PFS (P=.02 HR 0.284, 95%CI 0.23-0.618). FCGR2A and FCGR3A polymorphisms had no significant influence on degree of neutropenia after R-CHOP. *Conclusion.* Our results indicate that FCGRs polymorphisms: FCGR2A: H/H and H/R, and FCGR3A: V/V were statistically significantly related to high CR rate but were not predictive for OS and PFS in DLBCL pts treated with R-CHOP, suggesting other mechanisms of elimination of lymphoma cells than ADCC via FcgRIIA and FcgRIIIA receptors.

0676

REDUCTION OF IMMUNE SUPPRESSION PLUS R/CHOP CHEMO-IMMUNOTHERAPY IN DLBCL PTLD SUBTYPE: LONG TERM ANALYSIS OF EFFICACY AND RENAL SAFETY DATA IN A SINGLE INSTITUTION STUDY

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Background. A feared complication of solid organ transplantation is the development of post transplant lymphoproliferative disorder (PTLD). There is little consensus to the optimal treatment of PTLD, but general treatment principles include first the reduction of immune suppression. If this fails it is recommended to add in immunotherapy in the form of single agent rituximab. Recent data suggest the overall survival of PTLD utilising rituximab as a single agent is 76% at one year. (Choquet *et al.* Blood 2005). However for the more aggressive end of the spectrum i.e. Monomorphic PTLD such as DLBCL, it may be necessary to combine Rituxmab plus chemotherapy to achieve rapid disease control. There are few long term data on the safety and efficacy of R/CHOP in the management of DLBCL in terms of outcome both for disease and renal related morbidity / mortality. *Aims.* The aim of our study was to analyse the outcome data (for both disease and renal morbidity / mortality) in our series of patients. *Methods.* From our series of 49 patients diagnosed with PTLD, 11/49 patients first received reduction of immune suppression (RI) followed by administration of R/CHOP analysed on an intention to treat basis. The presence of latent EBV infection was determined by EBER *in situ* hybridization, and expression of EBV-LMP1 was detected by immunohistochemistry. Immunosuppressive protocols at our institution comprised prednisolone, Azathioprine or MMF, and Cyclosporin or Tacrolimus, all with Basiliximab induction from 2001 onwards. Routine depleting antibody therapy was not given at induction. *Results.* All patients had histological evidence of diffuse large B cell lymphoma (DLBCL) on the basis of morphology and immuno-histochemistry. EBV EBER *in situ* analysis was available in 8/11 patients of which 2/8 were positive. All patients first had reduction of immunesuppression (RI) and received R/CHOP. 4/11 were female; median age 48.5 years (range 27-55 years) and 7/11 were male; median age range 40 years (range 22-56 years). Development of PTLD occurred at a median of 10 years (range 1-20 years) of which 1/11was early (<12 months) and 10/11 were late (> 12 months). Median age at transplant was 38 years. Renal morbidity observed included 3 episodes of cellular rejection in 2 patients resulting in the loss of one allograft at 1.75 years but no functional change in the others. 4 patients returned to dialysis having lost their renal allograft function due either to rejection or to continued decline in already prejudiced allograft function. 10/11 patients entered CR after 6 cycles of R/CHOP. At a median follow up of 48 months (range 4-98) 3 year progression free survival was 81% and the 3 year overall survival 71%. 3/11 patients have died 1 of relapse, 1 of progressive disease and the other of myocardial infract (whilst in remission). *Conclusions.* These data show that a high proportion of PTLD patients with aggressive histology (DLBCL) can be successfully treated with RI and R/CHOP chemo-immunotherapy with an acceptable patient and renal allograft toxicity profile. 6/11 patients have been in CR for greater than 5 years treated with RI and subsequent R/CHOP chemo-immunotherapy.

0677

ONLINE REGISTRY OF DIFFUSE LARGE B-CELL LYMPHOMA IN SOUTH KOREA

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Background. There has been few nation-wide data regarding diffuse large B cell lymphoma (DLBCL) the most prevalent subtype of non-Hodgkin's lymphoma in South Korea. *Aims.* This study was aimed at survey on clinical spectrum of DLBCL in terms of incidence, prevalence in age, clinical characteristic, stage, and international prognostic index (IPI) as well as treatment outcomes. *Methods.* In 2007-2008, thirteen university hospitals evenly distributed in Korean peninsula participated in online registry of DLBCL at www.lymphoma.or.kr provided by Korean Society of Haematology Lymphoma Working Party and filed a total of 1,649 cases of DLBCL experienced in 1990-2008. The data was collected and analyzed by one institution. *Results.* Data showed steadily increasing annual incidence of DLBCL with slight male predominance (M:F=943:706) as well as linear correlation between age and incidence until the peak at ages of 65-70. Histologic transformation or combination with low grade was found in 33 cases (2%). Patients' performance was 1 or less on ECOG scale in 1,385 (84%). DLBCL presented among 977 cases in which primary site was identifiable, as primary nodular disease in 458 (47%) or extanodular disease in 519 (53%) with origins from stomach in 456 (12%), GI tract other than stomach in 57 (11%), CNS in 73 (14%), and bone in 10 (2%). Rare types of DLBCL included mediastinal lymphoma (n=10), intravascular lymphoma (n=4), pyothorax associated lymphoma (n=2), plasmablastic lymphoma (n=2), and primary effusion lymphoma (n=1). Among 1,446 patients in whom staging workup was completely filed, DLBCL presented at early disease in 63% (405 in stage I and 501 in stage II) or advanced disease in 37% (261 in stage III and 279 in stage IV). B symptoms accompanied in 189 (13%) and tumour deposit in marrow was documented in 78 (5%). Grouping according to IPI score found 795 (55%) in 0/1, 376 (26%) in 2, 188 (13%) in 3, and 87 (6%) in 4/5. Age adjusted IPI score was 0 in 506 (35%), 1 in 607 (42%), 2 in 275 (19%), 3 in 58 (4%). HBsAg was positive in 113 of 813 patients (14%), anti-HCV positive in 17 of 810 (2%), anti-HIV positive in 3 of 750 (0.4%). Treatment including CHOP or R-CHOP therapy with or without radiotherapy was carried out in 1544 (94%). No measures were taken for various reasons or measures taken were unidentifiable in 105 (6%). Among those who had been treated, 993 (64%) were alive, free of disease in 80% of them, 417 were dead (27%), disease free in 13% of them, and 144 (9%) were lost to follow-up, disease free in 23% of them. Age < 60, stage on diagnosis, IPI score regardless age adjusted or not, and addition of rituximab to CHOP therapy, were shown to have given statistically meaningful impact on survival duration in all. Summary/*Conclusions.* The epidemiology, clinical spectrum, and biological behaviour of DLBCL in Korea are similar to those of western countries, and the incidence is continuously increasing.

0678

CLINICAL OUTCOMES AND PROGNOSTIC FACTORS IN PATIENTS WITH BREAST DIFFUSE LARGE B CELL LYMPHOMA; CONSORTIUM FOR IMPROVING SURVIVAL OF LYMPHOMA (CISL) STUDY

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Background. The breast is a rare extranodal site of non-Hodgkin lymphoma, and primary breast lymphoma (PBL) has been arbitrarily defined as disease localized to one or both breasts with or without regional lymph nodes involvement. *Aims.* The aim of this study was to evaluate the clinical outcomes in patients with diffuse large B cell lymphoma (DLBCL) and breast involvement, and to find the criteria of PBL reflecting the outcome and prognosis. *Methods.* We retrospectively analyzed data from 68 patients, newly diagnosed with DLBCL and breast involvement at 16 Korean institutions between January 1994 and June 2009. Results. The median age at diagnosis was 48 years (range, 20-83 years) and all patients were female. Forty-three (63.2%) patients were PBL according to previous arbitrary criteria, sixteen (23.5%) patients were classified as high-intermediate to high risk according to the international prognostic index (IPI). According to the number of involved extranodal disease, the patients with one extranodal disease in the breast (OEND) with or without nodal disease were 49 (72.1%), and those with multiple extranodal disease (MEND) were 19 (27.9%). Sixty-seven (98.5%) patients were treated with systemic chemotherapy, 66 (97.1%) treated with anthracycline-based regimens. Fifty-five (80.9%) patients were treated with four or more than four cycles of systemic chemotherapy with or without any local treatment modalities such as surgery or radiotherapy. Any surgery or radiotherapy to the breasts was performed in 23 (33.8%) and 21 (30.9%) patients, respectively. Among 23 patients treated with surgery, modified radical mastectomy was performed in 13 (56.5%) patients. Forty-two (61.8%) patients were received chemoimmunotherapy with rituximab. During median follow-up of 41.5 months (range, 2.4-186.0 months), estimated 5-year progression-free survival (PFS) and overall survival (OS) were 53.7% (95% Confidence Interval [CI], 46.1-61.3) and 60.3% (95% CI, 53.1-67.5), respectively. The 5-year PFS and OS was significantly higher for patients with the OEND group than those with the MEND group (5-year PFS, 64.9% [95% CI, 56.0-73.8] *vs.* 27.5% [16.1-38.9], P=0.001; 5-year OS, 74.3% [66.7-81.9] *vs.* 24.5% [11.5-37.5], P<0.001). In univariate analysis, Ann Arbor stage III or IV (P=0.010), ECOG performance status of 2 or 3 (P=0.015), elevated levels of LDH (P=0.049), high-intermediate to high IPI (P=0.001), SBL group according to previous criteria (P=0.008), the MEND group (P<0.001), and fewer than four cycles of systemic chemotherapy (P<0.001) were significantly associated with lower OS. In multivariate analysis, MEND (hazard ratio [HR], 3.61; 95% CI, 1.07-12.2) and fewer than four cycles of systemic chemotherapy with or without local treatments (HR, 4.47; 95% CI, 1.54-12.96) were independent prognostic factors for worse OS. Twenty-five (36.8%) patients experienced progression, and the cumulative incidence of progression in multiple extranodal sites or other than breasts and central nervous system was significantly different between the OEND group and the MEND group (5-year cumulative incidence, 9.7% [4.3-15.1] *vs.* 49.0% [33.9-64.1], P=0.001). Conclusions. Our results show that the patients included in OEND group, reflecting different treatment outcome, prognosis and pattern of progression, should be considered as PBL in the future trial. Further studies are warranted to validate our suggested criteria.

0679

THE TREATMENT MODIFICATION DOES NOT SEEM TO CHANGE THE DLBCL PATIENT´S OUTCOME WHEN CORRELATED WITH EARLY PET RESULTS

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Background. It has been demonstrated that early PET examination can have prognostic impact in DLBCL. There is however only scarce data on the treatment modification based on early PET results. We present the retrospective analysis of the treatment modification in correlation with early PET results and its impact on patient´s outcome. Patients and *Methods.* The inclusion criteria were confirmed DLBCL, curative treatment and early PET (after 2nd or 3rd) cycle. The PET was not intended for treatment change, but the treating physician knew the results. Patients were recruited out of consecutively treated patients in one center (n 102) (dg IV/2000-IV/2009) and out of prospective phase II trial (ASH 2007, abstr 21, n 51). PET was interpreted as positive or negative. As a treatment modification was considered unplanned unplanned additional therapy or significant reduction of planned treatment. The endpoints were the PFS and OS according to the consensus definition (2007). *Results.* The median age of pts was 53 y (18-80), median follow up was 28 months, the aaIPI distribution was as follows: low 17 (11.1%), low-intermed 19 (12.4%), intermed-igh 67 (43.8%), high 50 (32.7%). All patients except 14 (9,2%) received rituximab. Seventy one (46.4%) pts were scheduled to intensive treatment protocol R-MegaCHOP/ESHAP/BEAM (Trneny, ASH 2007), 6 pts (3.9%) to CHOP followed by ASCT, 73 pts (47.8%) to CHOP regimen, and 3 ot other anthracyclin regimens. The early PET was negative in 104 pts (68%) and positive in 49 pts (32%). The 3 y PFS was 87.7% for PET neg and 56.1% for PET pos resp. (P 0.001) and 3y OS 91.9% and 68.6% resp. (p 0.001). 29 (80.2%) pts out of 36 previously PET pos pts who were examined at the end became PET negative. There was nonsignficant trend for better PFS (p 0.09) and for better OS (P 0.06) for PET neg-neg patients compared to the pos-neg patients. Compared to the preplanned therapy no change was in 107 cases (69.9%), the therapy was decreased in 20 cases (13.1%), and additional therapy was used in 26 cases (17%). The reduction of therapy consisted out of skipping of ASCT (10 cases), RT (6 cases) and significant chemotherapy reduction (4 cases), the additional therapy consisted out of RT (19 cases), ASCT (7 cases) and maintenance therapy (10 cases). The 3 y probability of early PET neg patients with unchanged and decreased treatment resp. were for PFS 87.7% *vs.* 100% resp. (p ns) and for OS 91.7% *vs.* 100% (p ns). The 3y probability of early PET pos pts with unchanged *vs.* additional treatment resp. was for PFS 57.7% *vs.* 66.7% resp. (P ns) and for OS 70.2% *vs.* 77.2% (P ns). *Conclusions.* We have confirmed prognostic value of early PET on outcome in this highly IPI poor prognostic group of pts (HI and H 76.5%). The reduction of treatment in early PET negative pts seems to have no adverse effect on outcome, on the other hand the additional treatment does not seem to improve the outcome of early PET positive patients.

0680

THE OUTCOME OF DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL) PATIENTS TREATED WITH R-CHOP IS NOT PREDICTED BY INTERIM EVALUATION OF 18-FDG-POSITRON EMISSION TOMOGRAPHY/COMPUTED TOMOGRAPHY (PET)

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Background. In DLBCL patients PET evaluation has a defined role to assess the final response, but the predictive value of interim PET is contradictory. Standardized criteria of interpretation of Interim PET have not been well established yet and the visual analysis of PET results by dichotomous evaluation as positive or negative is difficult to apply. Aim of study. to determine the predictive value of interim (I-PET) and final PET (F-PET) on Progression Free Survival (PFS) in DLBCL patients treated with R-CHOP. Patients and *Methods.* From April 2004 to December 2008, 82 newly diagnosed DLBCL patients treated in five Hematology Departments were included. Clinical features were as follows: median age 56 years (19-81); 42 males and 40 females, 29 stage I-II and 53 stage III-IV; 47 L/LI and 35 I/IH IPI score. All patients were treated according to the planned therapy, not modified by I-PET results, with 6-8 R-CHOP. IF-RT was planned to areas of bulky disease and given to 13 patients. All patients had PET scan performed at the diagnosis, during treatment (I-PET) and at the end of therapy (F-PET). All PET results were centrally reviewed and defined as positive or negative by visual dichotomous response criteria according to the First Consensus Conference (Deauville 2009). *Results.* All patients were evaluable for response. I-PET was performed after two R-CHOP in 46 pts, after three in 13 and after four in 23. At the end of therapy 73 pts (89%) achieved a CR and nine (11%) were non responders. Fifty-five patients (67%) were negative and 27 (33%) positive at the I-PET; 69 pts (84%) were negative and 13 (16%) positive at the F-PET. 15/27 (56%) I-PET positive patients became F-PET negative at the end of the therapy. The concordance between clinical CR and F-PET negativity was 99%: one CR pt was false F-PET positive due to parothid carcinoma. The prognostic impact of PET results on the outcome was evaluated. With a median FU of 18 months, Progression-free survival (PFS) was 80%. PFS did not correlate with I-PET results, 18-months PFS rates were: 84% in I-PET negative patients and 74%in I-PET positive patients (p .198) (Figure 1A). Conversely F-PET strongly predicted PFS (p .015): 84% in F-PET negative patients and 61% in F-PET positive patients. (Figure 1B). A further analysis for progression was performed to adjust the effect of I-PET analysis for other known risk factors (age ≥60, stage, PS, IPI, LDH, bulky, number of extranodal sites, Bone Marrow involvement): only LDH (p .005) and IPI 0-2 *vs.* 3-5 (p .001) were confirmed as independent predictors for progression. The use of G-CSF during treatment did not influenced I-PET results. *Conclusions.* Our results indicate that in DLBCL patients treated with R-CHOP a positive interim PET did not predict a worse outcome. Conversely, final PET results strongly correlate with PFS. A longer follow up is necessary to validate our data. Prospective larger studies and standarditation of criteria for interim PET are needed to assess the prognostic value of interim PET in DLBCL patients.

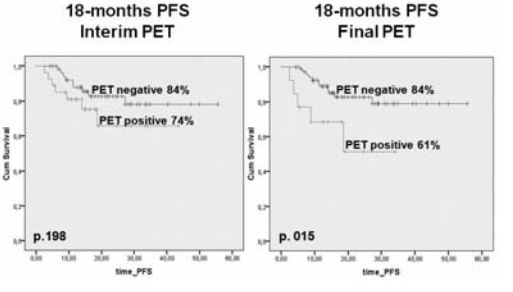

Figure 1. Median follow-up 18 months.

0681

AGGRESSIVE CRANIOFACIAL LYMPHOMA IN THE PRE-RITXUIMAB AND RITUXIMAB ERA

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Background. Among 4165 patients treated in the NHL-B1, NHL-B2, High-CHOEP, Mega-CHOEP, RICOVER-60 and MInT trials of the German High Grade Non-Hodgkin Lymphoma Study Group (DSHNHL), 316 patients had craniofacial involvement by their lymphoma. *Aims.* To analyze clinical characteristics and response to therapy of 316 patients with craniofacial lymphomas treated with CHOP-like chemotherapy with and without rituximab. *Methods.* A retrospective analysis was performed to compare characteristics and treatment outcome of patients with craniofacial lymphoma to all the other patients of these trials. *Results.* Of the 316 patients, 32 had orbital, 101 paranasal sinus, 44 main nasal cav-

ity, 109 mouth, 28 tongue and 57 salivary gland involvement. Patients with craniofacial lymphoma presented with a significantly lower LDH, better performance status, lower Ann Arbor Stage and less bulky disease compared to the entire population (elevated LDH: 16.8% *vs.* 35.9%, P=<0.001; ECOG >1: 2.8% *vs.* 10.1%, P=<0.001; stage III/IV: 28.2% *vs.* 40.7%, P=<0.001; bulky disease: 14.9% *vs.* 39.3%, P=<0.001). 3-year event free survival (70.4% *vs.* 60.5%, P=<0.001) and 3-year overall survival (81.2% *vs.* 75.6%; P=0.044) were better in patients with craniofacial involvement compared to the entire population of these trials. Multivariate analysis confirmed all IPI-relevant risk factors as significant and independent. With respect to different treatment strategies, the addition of rituximab to CHOP-14 in the RICOVER-60 trial reduced the relative risk to 0.6 for EFS and 0.5 for OS. This is clinically very relevant, but not significant due to the limited number of patients. In contrast, additive radiotherapy to the craniofacial involvement for patients in CR/CRu or PR at the end chemo- or immunochemotherapy, which was given to 178 patients had no influence on the outcome of these patients compared to patients not receiving additive radiotherapy (n=68). The relative risk for EFS was 1.0 (P=0.863) and 1.5 (P=0.315) for OS in patients receiving additional radiotherapy compared to patients receiving systemic therapy only. *Conclusions.* Patients with craniofacial lymphoma present with favorable prognostic parameters and treatment results are better in this population compared to the whole population of patients with aggressive lymphomas. Our results do not support the addition of radiotherapy to systemic therapy in these patients.

0682

FOLLOW UP WITH 18-FDG PET/CT IN AGGRESSIVE LYMPHOMAS IN FIRST PET-NEGATIVE COMPLETE REMISSION

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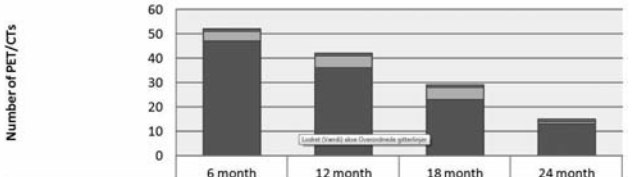
Background. The use of 18-FDG-PET/CT (PET/CT) is well established as a primary investigation, interim scan and post-therapy evaluation of aggressive non-Hodgkin's lymphomas. However, there has been little focus on its value for disease surveillance. *Aims.* To evaluate use of PET/CT scans during follow-up in asymptomatic patients successfully treated with first-line therapy for aggressive non-Hodgkin's lymphoma (NHL). *Methods.* We retrospectively analyzed patients with aggressive NHL treated at our institution from 2006 to 2009. Patients were eligible if they achieved a PET/CT based complete remission (CR) on primary treatment and if PET/CT was used approximately every 6 month during the first two years of follow-up. Only PET/CTs performed in patients with no clinical or biochemical signs of relapse were included, whereas PET/CTs performed in patients with suspected lymphoma relapse were not analyzed. PET/CTs were described as true positive, false positive, true negative and false negative on a basis of PET/CT result combined with biopsy results, subsequent imaging or clinical course. For each positive scan, maximum standardized uptake value (SUVmax) was determined. *Results.* Of a total of 80 patients in CR after first-line therapy for aggressive NHL during the time period, 52 were eligible (43 Diffuse Large B-cell lymphoma, two Burkitt Lymphoma, one grade III Follicular Lymphoma and six T-cell Lymphomas). Median age was 61 years and median follow up 18 months. Each patient had between one and four PET/CTs giving a total number of 138 PET/CT's. In four cases relapses were detected by PET/CT in patients regarded CR at clinical evaluation. In one case the relapse was clearly visible on PET but with only discrete changes on CT. Four PET/CTs (3%) were true positive and 119 PET/CTs (86%) were true negative. The numbers of false positive and negative PET/CT's were 15 (11%) and 0 respectively (Figure 1).



	6 month	12 month	18 month	24 month
Total number of PET/CTs	52	42	29	15
False negative	0	0	0	0
True positive	1	1	1	1
False positive	4	5	5	1
True negative	47	36	23	13

Figure 1. PET/CT results at six month intervals after end of therapy.

Specificity was 89% and sensitivity 100%. The predictive value of a positive and negative PET/CT was 21% and 100% respectively. The median SUVmax in false positive PET/CTs was 6.3 (range 2.1-12.8) and for true positive 10.2 (range 5.8-14). *Conclusions.* The most important conclusion is that a negative PET/CT strongly indicated absence of lymphoma. However the significant number of false positive PET/CTs consequently leads to patient anxiety and unnecessary biopsies. High SUVmax was seen more often in lymphoma but with an overlap to non-lymphoma lesions. Given the low number of unsuspected relapses found by PET/CT compared to the total number of PET/CTs performed, a question of poor cost effectiveness could be raised as PET/CT is an expensive procedure compared with CT. In only one case, did the PET procedure of the PET/CT play an important role in detecting relapse.

0683

PROGNOSTIC VALUE OF CEREBROSPINAL FLUID FLOW CYTOMETRY ANALYSIS IN AGGRESSIVE NHL PATIENTS AT HIGH RISK FOR LEPTOMENINGEAL DISEASE: FCM+ MAY PREDICT AN HIGH RISK OF CNS RELAPSE

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Background. In aggressive NHL intrathecal prophylaxis (IT-P) reduces the incidence of CNS relapse but may increased the toxicity of systemic chemotherapy. The identification of patients subgroups for whom IT-P may benefit is therefore important. Flow cytometry (FCM) assessment of cerebrospinal fluid (CSF) has recently been known to increase the proportion of positive cases with leptomeningeal disease in comparison to conventional cytologic examination (CC). However it's still unknown its prognostic value. *Aims.* The primary aim of this prospective, multicenter trial was to compare CC *vs.* FCM in a large cohort of NHL patients at high risk for LD. The secondary aim was to assessed the impact of FCM^+ on PFS and OS. *Methods.* Patients, with no evidence or signs of neurological disease, were enrolled if they were diffuse large B-cell lymphoma (DLBCL) with IPI 2-3, elevated LDH along with at least two extranodal sites or with bone marrow, testis, palate or paravertebral involvement; Burkitt lymphoma (BL); blastoid variant of mantle-cell lymphoma (B-MCL); B-cell precursor lymphoblastic lymphoma (B-LL); HIV^+ patients. Pts received IT-P standard with MTX +/- cytarabin except for liposomial cytarabin in BL. PFS was defined as the time from diagnosis to any type of progression or death from any cause and compared by the log-rank test.

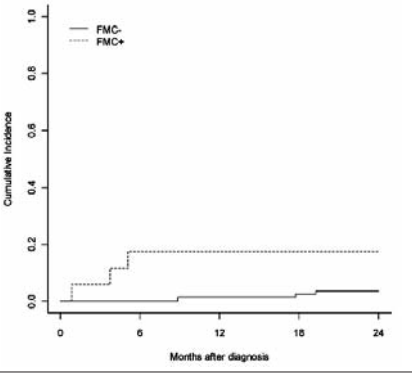

Figure 1. Cumulative incidence of CNS progression by FMC test result, accounting for competing events (death for any cause).

Results. From August 2004 to June 2008, 145 patients were enrolled by 12 centres. Clinical characteristics were: 96 males, median age 55 years (IQR:43-63); 111 patients (76%) with DLBCL, 22 pts (15%) with BL, seven pts (5%) with B-MCL and five pts (3%) with B-LL. Twenty-seven patients (19%) were HIV^+. FCM was able to detect a clonal population in 17 out of 145 patients (12%) whereas CC detected abnormal cells only among 7 pts (5%) (P=0.0002). Therefore, 10 patients (7%) were discordant: FCM^+/CC^-. From date of diagnosis, overall median follow up of survivors was 26 months. We observed 39 (30%) systemic progressions, 6 (5%) CNS progressions and 32 (25%) deaths. Among the 17 pts FCM positive, we observed 10 (59%) systemic progression,

three (18%) CNS progression (in two cases the disease of CNS was isolated whereas one pts presented a CNS progression along with systemic progression) and eight (47%) deaths (6 PD, 2 missing). PFS at 1 year was 71.2% (95%CI:62.1-78.5) in the whole group of patients. The progression risk was significantly higher in patients FCM^+/CC^+ compared with patients FCM^-/CC^- (P=0.003). An higher but not significant risk of progression was found in FCM^+/CC^- with respect to patients FCM^-/CC^-. At 24 months the cumulative incidence of CNS progression by FMC test result, accounting for competing events, was statistically higher in FCM^+ respect to FCM^- patients (18% *vs.* 4%)(P=0.010). *Conclusion.* FCM assessment of CSF is more sensitive than CC for detection of LD, but it's clinical relevance is still to be clearly defined. Our preliminary data suggest that patients FCM^+/CC^+ have an higher risk of progression compared with those FCM^-/CC^-, whereas discordant cases seem to have an intermediate prognosis. Moreover, pts FCM^+ seems to have an higher risk of CNS relapse in comparison to patients FCM^-.

0684

RITUXIMAB SERUM LEVELS DURING INDUCTION AND MAINTENANCE TREATMENT ARE DIFFERENT IN MEN AND WOMEN

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Introduction. Rituximab is one of the pivotal substances in the treatment of B-cell lymphoma. Little is known regarding rituximab serum levels. Materials and *Methods.* We conducted a prospective multicenter trial in patients with previously untreated follicular lymphoma stage III or IV, which required therapy. Primary endpoint was conversion of BCL2/IgH PCR positivity to negativity. One of the secondary endpoints was serum rituximab level. Induction therapy (R-FM) consisted of 6 cycles of rituximab 375 mg/qm i.v. day 1, mitoxantrone 10 mg/qm i.v. day 1, fludarabine 25 mg/qm i.v. days 2-4 recycling every 28 days for 6 cycles. Patients not progressing during induction therapy received maintenance rituximab 375 mg/qm every 8 weeks for 12 doses which was an off label use. All patients signed a written informed consent.

Table 1.

R-FM Induction (Q 4 weeks)

		Cycle 1			Cycle 2			Cycle 4			Cycle 6		
pre-dose		min.	median	max.	min.	median	max.	min.	median	max.	min.	median	max.
female	[ng/ml]		< 500		< 500	27.980	44.676	31.132	65.764	99.207	43.271	67.245	113.661
male	[ng/ml]		< 500		< 500	11.969	17.429	25.308	39.297	78.095	47.706	64.260	208.359
male	%					**44%**	**39%**	**81%**	**60%**	**84%**	**110%**	**96%**	**183%**
post-dose													
female	[ng/ml]	44.870	167.477	239.709	173.093	214.616	300.595	178.078	273.768	346.227	205.534	266.648	370.262
male	[ng/ml]	109.562	149.231	228.088	146.405	188.823	254.271	179.659	204.811	321.222	196.358	238.833	364.960
male	%	**244%**	**89%**	**95%**	**85%**	**88%**	**78%**	**99%**	**75%**	**93%**	**96%**	**93%**	**99%**
female		N = 10			N = 9			N = 7			N = 8		
male		N = 8			N = 9			N = 8			N = 6		

Interval 8 weeks

R - Maintenance (Q 2 months)

		Cycle 1			Cycle 2			Cycle 4			Cycle 6		
pre-dose		min.	median	max.	min.	median	max.	min.	median	max.	min.	median	max.
female	[ng/ml]	14.701	47.616	113.709	6.233	26.239	72.874	16.018	43.314	281.289	16.273	29.781	36.944
male	[ng/ml]	13.828	34.899	64.709	6.374	24.606	57.485	1.545	27.882	54.069	14.172	34.116	56.070
male	%	**94%**	**73%**	**57%**	**102%**	**68%**	**79%**	**10%**	**64%**	**21%**	**87%**	**127%**	**144%**
post-dose													
female	[ng/ml]	208.512	249.731	364.689	198.003	241.088	292.735	222.655	238.582	280.960	212.096	249.280	281.487
male	[ng/ml]	136.665	212.283	332.802	188.707	232.466	350.704	169.648	214.281	236.020	187.455	187.455	187.455
male	%	**66%**	**85%**	**91%**	**95%**	**97%**	**120%**	**99%**	**90%**	**84%**	**88%**	**75%**	**73%**
female		N = 9			N = 8			N = 7			N = 4		
male		N = 7			N = 5			N = 6			N = 5		

Results. Twenty-seven patients were entered in the study. Median age was 55 years, (range 32-73 years). Rituximab serum concentrations were measured before and after rituximab infusion during induction and maintenance. Rituximab serum levels during induction and maintenance treatment were significant higher in female than in male patients (Table 1). Induction R-FM resulted in 11 (91.7%) and 6 (54.5%) complete remissions, 3 (27.3%) and 0 partial remissions in female and male patients, respectively (P=.142). Event free survival after 36 months was .823 and .671 for female and male patients, respectively (P=0.33). Overall survival after 36 month was 1.0 and .71 for female and male patients, respectively (P=.06). Discussion. A rituximab dose of 375 mg/qm achieves significant higher serum levels in female than in male patients. These higher levels did not result in significant higher remission rates, event free survival or overall survival. There are several explanations for these findings. Patient numbers may be too small; observation period could be too short to find a difference that really exists. Rituximab levels in male are high enough and higher levels do not add something to the effect of the therapy.

0685

DOSE-DENSE THERAPY WITH NON-PEGYLATED LIPOSOMAL DOXORUBICIN (R-COMP 14 *VS.* 21) IS FEASIBLE AND EFFECTIVE FOR ELDERLY PATIENTS WITH NEWLY DIAGNOSED AGGRESIVE B-CELL NON-HODGKIN LYMPHOMA

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Background. The optimal treatment for elderly patients with non-Hodgkin's lymphoma (NHL) is still a matter of debate. The favourable toxicity profile of nonpegylated liposomal doxorubicin (Myocet™) makes its use particularly appealing for frail and elderly NHL patients. *Aims.* To evaluate the toxicity and the efficacy of nonpegylated liposomal doxorubicin when substituted for conventional doxorubicin in the CHOP 14 or 21 regimen for the treatment of elderly patients with newly diagnosed B-cell NHL. *Methods.* 69 patients with B cell NHL at diagnosis were split in 2 groups according to the Multidimensional Geriatric Assessment (MDGA). Patients with an Activities of Daily Living (ADL) = 6 received dose-dense R-COMP every 2 weeks (33 patients), whereas patients with an ADL < 6 received R-COMP every 3 weeks (39 patients). The median age was 70 years (range: 65-79) in the R-COMP 14 group and 75 years (range: 64-89) in the R-COMP 21. At baseline 23/33 (70%) patients had stage IV disease in the R-COMP 14 group, whereas 19/39 (49%) in the R-COMP 21. Median performance status and median number of comorbities were comparable between the 2 groups. Eighteen out of 33 (55%) patients had an intermediate or high risk International Prognostic Index score in the R-COMP 14 group, compared to 18/39 (46%) in the R-COMP 21. The median left ventricular ejection fraction (LVEF) before starting chemotherapy was comparable between the two groups (59% *vs.* 60%). *Results.* A total of 397 cycles of chemotherapy were administered (179 R-COMP 14 and 218 R-COMP 21). Grade 3/4 neutropenia occurred in 12% and 24% of cycles in the R-COMP 14 and 21 groups respectively, with an incidence of febrile neutropenia of 2% and 7% respectively. The relative dose intensity for the regimens was 93% for the R-COMP 14 group and 90% for the R-COMP 21 group, respectively. Regarding cardiotoxicity, only 2/33 patients presented a grade II-IV WHO toxicity in the R-COMP 14 group, whereas 5/39 in the R-COMP 21 group. All patients are evaluable for response. In the R-COMP 14 group, 23/33 patients (70%) obtained a CR, 8/33 (24%) achieved a PR, and 2/33 (6%) did not respond to therapy. In the R-COMP 21 group, 28/39 patients (72%) obtained a CR, 8/39 (21%) achieved a PR, and 3/39 (7%) did not respond to therapy. With a median follow-up of 10 months (range 2-20) and 10 (range 2-27) as of January 2010, 25/33 patients (76%) and 31/39 (79%) are alive and disease free in the R-COMP 14 and in the R-COMP 21 group, respectively. *Conclusions.* The stratification of patients according to the MDGA allows elderly and fit patients with aggressive B-cell NHL with poor prognosis (high IPI score) to receive dose dense chemotherapy which might favourably impact on response rate and survival. Due to the fact that the trial was design to demonstrate the superiority of the dose-dense regimen in terms of disease-free survival at 3 years, a longer follow-up is warranted to better define the impact of the R-COMP 14 regimen on the outcome of elderly patients with aggressive NHL.

0686

LENALIDOMIDE HAS CLINICAL ACTIVITY AND INDUCES DURABLE RESPONSES IN PATIENTS WITH TRANSFORMED LYMPHOMA: SUBSET ANALYSIS OF AN INTERNATIONAL PHASE 2 STUDY (NHL-003) IN AGGRESSIVE NON-HODGKIN'S LYMPHOMA

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Background. The heterogeneity among patients who transform from an indolent to an aggressive non-Hodgkin's lymphoma (aNHL) has led to a reported median survival ranging from 2.5 to 22 months. At a median follow-up of 9 years after initial diagnosis there appears to be a transformation risk of ~30% at 10 years (Al-Tourah JCO 2008). A retrospective analysis of 600 patients with aNHL showed that the 10-year overall survival was significantly longer for non-transformed patients (75%) *vs.* transformed patients (36%; P<0.001). Despite the aggressive nature of transformed NHL there are few prospective studies available to guide therapeutic decision for this patient population. The few trials that include patients with transformed lymphoma (TL) show limited activity with treatments used for other NHLs. Lenalidomide is an immunomodulatory agent that also exhibits tumoricidal activity in *in vitro* models of both aggressive and indolent NHL. Here we report on a subset of patients with TL, who were enrolled in a large international phase 2 trial of lenalidomide monotherapy in the setting of relapsed/refractory aNHL (off-label). *Aims.* To determine the clinical benefit and safety of single-agent lenalidomide in patients with relapsed/refractory TL. *Methods.* As part of the inclusion criteria for this multicenter, single-arm, open-label study, patients were required to be ≥18 years old, have a good performance status, have had ≥1 prior therapy, and ≥1 measurable lesion of ≥2 cm. Patients provided informed consent. Oral lenalidomide 25 mg/day was given on days 1-21 of a 28-day cycle, and continued until disease progression or unacceptable toxicity. Response rate was the primary endpoint, and secondary endpoints included response duration, time to progression, progression-free survival (PFS), and safety. Pathology assessment and efficacy evaluation were as per investigator assessment. *Results.* 217 patients enrolled in the study and received lenalidomide. Of these, 33 patients (15%) had TL; 23 had transformed follicular lymphoma (tFL), 7 had transformed chronic lymphocytic leukemia/ small lymphocytic leukemia (tCLL/SLL), and 3 were other or unknown. For patients with TL, the median age was 66 years (42-84), 58% of patients had stage IV disease, patients had a median of 4 (1-12) prior therapies, and 24% had received prior stem cell transplantation. Responses were observed in 45% of patients with TL, which included 21% complete response (CR)/CR unconfirmed. Responses to lenalidomide were noted in 13 of 23 (57%) patients with tFL and 0 of 7 patients with tCLL/SLL. The median PFS was 5.4 months and at a 5.6-month median follow-up the median response duration was 12.8 months. Reversible myelosuppression was the most common grade 3 or 4 adverse event. The occurrence of neutropenia (33% grade 3; 15% grade 4) and thrombocytopenia (12% grade 3; 3% grade 4) was consistent with results observed in other studies of lenalidomide in NHL. *Conclusions.* This study demonstrates that lenalidomide monotherapy has promising clinical activity and can achieve durable responses in pretreated patients with TL. The variation in responses to lenalidomide for patients with tFL and tCLL/SLL suggests that the original histology may be a factor in the response to treatment.

0687

SINGLE-AGENT LENALIDOMIDE FOR THE TREATMENT OF RELAPSED OR REFRACTORY AGGRESSIVE LYMPHOMA PATIENTS

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Background. Aggressive lymphoma comprises a heterogeneous group of B-cell malignancies, each characterized by a unique histology and molecular markers. Although initial therapy can be effective, up to 40% of patients with DLBCL either fail to respond or relapse after initial therapy (Habermann TM *et al.* JCO 2006), and relapses are likely among patients with MCL. For such patients survival outcomes are particularly poor with few effective treatment options available. Lenalidomide is an immunomodulatory agent, which also exerts both antiproliferative and tumoricidal effects. In relapsed or refractory aggressive lymphoma this therapy proved to be a good treatment option with a ORR of 35%, which included 12% of patients with a complete response (CR), and a median duration of response (DR) lasting 10.4 months (Wiernik *et al.*, JCO 2008). *Aims.* In this prospective study we evaluate the safety and efficacy of lenalidomide oral monotherapy in patients with relapsed or refractory aggressive lymphoma. Mhetods. Patients were required to have relapsed or refractory aggressive lymphoma after ≥2 prior therapies. We treated with oral lenalidomide 25 mg once daily on days 1 to 21, every 28 days, for 52 weeks, until disease progression or intolerance. The primary end point was the ORR, feasibility and the safety. Response was evaluated after 4 cycles or progression of disease. *Results.* Starting from October 2008 we have treated 22 patients with a median age of 66 years (range 53-75). The histotype were: 14 diffuse large B-cell lymphoma (64%), 4 mantle cell lymphoma (18%), 2 follicular lymphoma (9%), 1 PTCL (4.5%) and 1 Hodgkin lymphoma (4.5%). Median time from diagnosis was 3 years (range 1-13) and patients received a median of 4 prior therapies (range 2-7); 13 patients (59%) were refractory to their last treatment, 8 (36%) patients were in partial remission and one patient (5%) was in complete remission. All patients but two used Rituximab. The patients performed a median of 2 cycles (range 1-8); two of them (9%) reduced dose of lenalidomide to 10 mg due to neutropenia grade IV. Five patients (23%) delayed treatment, 4 for neutropenia grade 3-4 and 1 due to herpes zooster reactivation. Seventeen patients were evaluable for response, five patients did not reached the fourth cycle. Three patients obtained a CR/CRu (17,5%), 3 a PR (17,5%) with an ORR of 35%. The most common grade 3-4 hematological adverse events were: neutropenia (32%), anemia (4.5%) and thrombocytopenia (4,5%). The extrahematological toxicities were: hypercalcemia (1), herpes zooster (1) and renal failure (1). Hypercalcemia was associated with progressive disease after fourth cycles. The median time duration to response was 1,5 months (range 0,5- 3,5 months). *Conclusion.* single agent lenalidomide appears to be active in heavily pretreated relapsed or refractory aggressive lymphoma with an ORR of 35%. Monotherapy lenalidomide was well tolerated in this group of patients; haematological toxicity represent the predominant adverse event, manageable with use of stimulating factors or delaying therapy.

Biology of thrombosis

0688

RIVAROXABAN CALIBRATOR AND CONTROL SETS MEASURING RIVAROXABAN PLASMA CONCENTRATIONS USING THE PROTHROMBIN TIME

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Background. Rivaroxaban is an oral, direct Factor Xa inhibitor which is marketed in more than 60 countries for the prevention of venous thromboembolism and is under development for the prevention and treatment of arterial and venous thromboembolic diseases. No routine coagulation monitoring is required, but the rivaroxaban plasma concentration might be needed in some cases, i.e. severe overdose or compliance. Variation in the response sensitivity of prothrombin time (PT) reagents to rivaroxaban is well described in the literature and the international normalized ration correction of results can not be used to correct for this variability. *Aims.* This multicentre study evaluated rivaroxaban calibrators and the PT for the measurement of rivaroxaban plasma concentrations. The interlaboratory precision of the measurement of rivaroxaban plasma concentrations (ng/mL) was also evaluated. *Methods.* 20 centres were provided with a set of rivaroxaban calibrators (0, 41, 219 and 430 ng/mL) and a set of unknown rivaroxaban pooled human plasma controls (19, 160 and 643 ng/mL). The evaluation was carried out over 10 consecutive days by each laboratory using its own PT reagent as well as STA® Neoplastine CI Plus, Diagnostica Stago. A rivaroxaban calibration curve was produced daily. The day-to-day precision was evaluated by testing in duplicate three plasma controls. The control was diluted and re-tested if the level was above the highest concentration of the calibration curve. *Results.* A large interlaboratory variation (in seconds) was shown for the controls when local PT reagents were used, and their coefficient of variation (CV) was 14 to 30%; but the results were more consistent when using the same PT reagent with a CV of <6% (with undiluted samples). Expressed in ng/mL, a smaller interlaboratory variation was observed (CV ranging from 2% for the highest to 7.5% for the lowest). In addition, the CV for the 41 ng/mL calibrator with the central reagent was 4.4%, and the results were reliable for concentrations >40 ng/mL and up to 600 ng/mL. Summary/*Conclusions.* Rivaroxaban measurements may be of assistance when determination of its plasma concentrations is required. The results indicate that the PT expressed in seconds cannot be used to measure rivaroxaban activity in plasma due to the large variation in the sensitivity of different reagents to rivaroxaban; Rivaroxaban concentrations can be measured with acceptable precision by using a rivaroxaban calibration curve with reliable estimation for concentrations >40 ng/mL using both local PT reagents and STA Neoplastine CT Plus. Further validation of these methods in plasma samples obtained from patients with specific clinical events such as hemorrhage, recurrent thromboembolism or suspected overdose.

0689

G-CSF ALONE OR IN COMBINATION WITH CHEMOTHERAPY DOES NOT MOBILIZE ENDOTHELIAL PROGENITOR CELLS INTO PERIPHERAL BLOOD

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Background. Increasing interest has been devoted to circulating Endothelial Progenitor Cells (EPCs) because of their possible use as a therapeutic tool in the treatment of vascular lesions of ischemic tissues or as a target for anti-neoplastic therapy. Several drugs can increase the number of EPCs into the peripheral blood (PB); however, there is insufficient data concerning the frequency of EPCs during hematopoietic stem/progenitor cells mobilization and leukapheresis procedures for transplantation purposes. *Aims.* To investigate EPC mobilization and collection both in patients and in healthy donors (HD) undergoing different regimen of $CD34^+$ cell mobilization for autologous or allogeneic transplantation, respectively. *Methods.* We studied 19 patients with hematological malignancies treated with chemotherapy and G-CSF (5 μg/Kg), and 18 healthy donors (HD) treated with G-CSF alone (5 μg/Kg), as mobilizing regimens. Twenty-four healthy non-mobilized adults (CTRLs) were included in the study. The informed consent was obtained from all the subjects. EPCs were detected the day of mobilization, both in the PB and in the leukapheresis samples. By flow cytometry, we evaluated $CD34^+CD45^-$ cells, including EPCs but also mature endothelial cells (ECs), and the subset of $CD34^+CD45^-$ cells co-expressing the CD133 and VEGFR-2 antigens ($CD34^+CD133^+VEGFR2^+CD45^-$), representing a population of putative EPCs. Results are expressed as median percentage (range) of electronically gated $CD34^+$ cells. We assessed the number of Endothelial Colony Forming Cells (ECFCs) grown *in vitro* according to Ingram *et al.* (Blood 2004;104:2752). Results are expressed as median number (range) of ECFC/107 mononuclear cells. *Results.* The percentage of $CD34^+CD45^-$ cells in the PB of HDs (10.9%, 1.1-25.3) was higher (P<0.0002) than that of patients mobilized with cyclophosphamide and G-CSF (0.6%, 0.1-7.3), and comparable to that of CTRLs (13.8%, 0-54.7). A significant difference between the HDs and patients was confirmed in leukapheresis samples (P=0.006). The percentage of $CD34^+CD133^+$ $VEGFR2^+CD45^-$ cells was increased in the PB of HDs receiving G-CSF (13.2%, 0-36.9) with respect to that found in the PB of patients receiving cyclophosphamide and G-CSF (0.0%, 0-25.9), and to that of CTRLs (0.0%, 0-83.4) but the difference was not statistically significant. Similar results were obtained in the leukapheresis samples (*data not shown*). The functional analysis of EPCs showed that the frequency of ECFCs, progenitors faithfully belonging to the endothelial lineage, was comparable both in the PB and in the leukapheresis of either groups, with a median value of 0/107 cells (0-1.47). These frequencies were not statistically different from that found in the PB of the CTRLs (0/107 cells, 0-0.5). Summary. Our results indicate that the frequency of $CD34^+CD45^-$ cells, including both EPCs and mature ECs, is significantly higher in subjects receiving G-CSF than in those receiving cyclophosphamide and G-CSF, and comparable to that of CTRLs. However, a more specific phenotypic and functional characterization of EPCs showed no significant difference within the two pharmacological treatments and CTRLs, suggesting that the increase of $CD34^+CD45^-$ cells in HD compared to patients is mainly due to mature ECs. These findings suggest that the mobilization of EPCs into the PB is regulated by pathways different from those involved in hematopoietic stem/progenitor cells.

0690

LUPUS ANTICOAGULANT TESTING IS UNINTERPRETABLE IN PATIENTS RECEIVING RIVAROXABAN THERAPY FOR VENOUS THROMBOEMBOLISM TREATMENT

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Background. Rivaroxaban a direct factor Xa inhibitor, is a member of an emerging class of anticougulants being evaluated in the treatment of venous thromboembolic (VTE) disease. Testing for a lupus anticoagulant (LA) is central in the clinicopathologic diagnosis of the antiphospholipid syndrome and thus is often employed to help guide the duration of anticoagulation following a VTE event. There is however, a paucity of literature regarding the effect of rivaroxaban on the coagulation based assays employed in identifying a LA. *Aim.* To evaluate the utility of LA testing in patients being treated with rivaroxaban for VTE. *Methods.* We identified 22 patients randomised to receive rivaroxaban on the EINSTEIN VTE trial on whom lupus anticoagulant testing was performed. The EINSTEIN trail is a randomised open label trial evaluating the efficacy and safety of rivaroxaban compared with warfarin in the treatment of acute symptomatic deep vein thrombosis or pulmonary embolism. Testing was performed while patients were taking a dose of 20 mg of rivaroxaban daily. Blood samples were taken within 12 hours of the last dose of rivaroxaban, but at no defined time interval from the last dose. Testing for a LA was performed using 3 screening assays and a confirmatory assay. The screening assays comprised a kaolin clotting time (KCT), dilute thromboplastin time (DTT) (Dade INNOVIN) at 1:100 and 1:200 dilutions and a dilute russell viper venom test (DRVVT) (Life Diagnostics). A confirmatory test to demonstrate phospholipid dependence of the inhibitor was performed using Stago Staclot LA™ (Bayer Diagnostics) assay. *Results.* Twenty-two patients were identi-

fied. The DRVVT was prolonged in 20/22 patients (range:1.1-3.4 mean: 2.33±1.5 2SD) (normal ratio:1.0-1.2). The DRVVT failed to correct upon performing a 50:50 mix with normal plasma in all but 1 patient with an initially elevated ratio. Similarly, the DTT at 1:100 and 1:200 dilutions also appeared to be sensitive to the presence of rivaroxaban evidenced by an increased ratio in 13/20 and 16/22 patients respectively (DTT 1:100 range:1.0-2.0 mean:1.21±0.45 2SD; DTT1;200 range:0.9-2.9 mean:1.46±0.82 2SD) (normal ratio:1.0-1.2). Conversely, the KCT was the least sensitive of the lupus anticoagulant screening tests to the presence of rivaroxaban, being normal in 20/22 patients. The Staclot LA™ confirmatory test for phospholipids dependence of a potential lupus inhibitor was performed in 13 patients and failed to demonstrate phospholipid correction in all patients tested. *Conclusion.* LA testing often plays an integral role in determining the duration of anticoagulation in patients with unprovoked VTE. LA testing using currently available laboratory screening tests whilst the patient is receiving rivaroxaban are uninterpretable. To confirm whether the patient has a LA, testing must be repeated when rivaroxaban has been ceased.

0691

GENETIC VARIABILITY OF THE G58A POLYMORPHISM ON FIBRINOGEN A-CHAIN GENE IN ADVANCED ATHEROSCLEROSIS: EFFECTS ON FIBRINOGEN, D-DIMERS AND PLASMINOGEN LEVELS

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Background. Evidence suggest that the G58A polymorphism on fibrinogen a-chain gene is associated with increased fibrinogen levels in healthy individuals. However, it is still unclear, whether this polymorphism is associated with coagulation/thrombosis in patients with coronary artery disease (CAD). In the present study we examined the impact of this polymorphism on fibrinogen levels, D-dimers levels and plasminogen levels. *Methods.* The study population consisted of 395 subjects, 246 of which angiographically documented for CAD. The G58A polymorphism was detected by polymerase chain reaction (PCR) and appropriate restriction enzymes. Fibrinogen levels were measured by immunonephelometry, while plasminogen and D-dimers levels were measured by standard coagulometry techniques. *Results.* The genotype distribution was GG: 37.8%, GA: 39.4% and AA: 22.8% for patients with CAD, while GG: 33.5%, GA: 44.3% and AA: 22.2% for controls. Patients with CAD had significantly higher fibrinogen levels (mg/dL) than controls (434.7±132.7 *vs.* 384.7±103.7, P=0.0002). However, in patients with CAD fibrinogen levels were not significant higher for 58AA homozygotes *vs.* 58G carriers (453.6±131.4 *vs.* 441.1±140.6, P=NS), while similar difference occurred in controls (AA: 385.2±129.4 *vs.* GG+GA: 392.6±103.0, P=NS). Moreover, D-dimers levels (mg/L) were significantly higher in CAD patients than controls (409.7±188.2 *vs.* 332.8±199.4, P<0.0001). In addition, there was a significant difference for 58G carriers *vs.* 58AA homozygotes for CAD patients (506.4±418.8 *vs.* 662.2±627.1, P<0.05), but not for controls (AA: 415.6±289.6 *vs.* GG+GA: 355.9±276.5, P=NS). Finally, CAD patients and controls had no significant difference in plasminogen levels (u/mL) (119.8±79.1 *vs.* 113.9±22.9, P=NS). Patients with CAD had no difference in plasminogen for 58AA homozygotes *vs.* 58G carriers (110.2±20.6 *vs.* 112.2±17.2, P=NS), while no significant difference was observed for controls (AA: 112.3±16.7 *vs.* GG+GA: 114.3±23.5, P=NS). *Conclusions.* Our findings indicate that the G58A polymorphism on fibrinogen a-chain gene affects D-dimers levels in patients with coronary artery disease. These findings provide a possible mechanism by which this polymorphism may affect thrombotic process/coagulation independently of fibrinogen levels and may have important clinical implications.

0692

LACK OF ASSOCIATION BETWEEN PLASMINOGEN ACTIVATOR INHIBITOR -1 4G/5G GENE PROMOTER POLYMORPHISM AND PAI-1 PLASMA LEVELS IN PATIENTS WITH ISCHEMIC STROKE

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Background and purpose. The insertion/deletion 4G/5G polymorphism at 675 pb, located before the initial transcriptional site at the promoter region of the plasminogen activator inhibitor - 1 (PAI-1) gene, has been associated with arterial disease. In this study, we investigated the frequency of this PAI-1 polymorphism and its association with PAI-1 and C-reactive protein (CRP) levels in young survival patients of Ischemic Stroke (IS). *Methods.* The plasma levels of PAI-1 and CRP and the frequency of 4G/5G polymorphism were analyzed in a group of 127 Brazilian patients that presented IS and in 201 healthy and unrelated control subjects. *Results.* The levels of CRP (P<0.001) and PAI-1 (P<0.001) were significantly higher in patients when compared with control group. However, after adjustments for sex, age, smoking and hypertension using a multivariate regression model, only PAI-1 plasma levels were independently associated with risk of IS (OR 3.40; 95% CI 1.49 - 7.74; P=0.001). The 4G/4G genotype was significantly more frequent among control subjects as compared to patients (OR 0.41; 95% CI 0.24 - 0.68; P<0.001). Furthermore, no significant correlations were detected among the PAI-1 polymorphism and PAI-1 plasma levels. *Conclusion.* The data obtained suggest that, although increased PAI-1 plasma levels are associated with development of IS in Brazilian young patients, they are not influenced by the 4G/5G PAI-1 polymorphism.

0693

QUANTITATIVE PLASMA D-DIMER LEVELS AMONG PATIENTS SUSPECTED FOR PULMONARY EMBOLISM AND DEEP VENOUS THROMBOSES

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Background. D-dimer tests are used in various diagnostic strategies to exclude pulmonary embolism and deep venous thromboses.However,their role as an exclusionary first-line test is still uncertain,mainly because accuracy of the test varies according to the assay and the studied population. Objective. To validate the observation that a low positive D-dimer level has a high negative predictive value for thromboembolic events diagnosis. Design and setting. A blinded retrospective study was conducted in all patients who had D-dimer values between 255-500ng/mL. The results of subsequent diagnostic radiological studies were then assessed for positive results. The notes for confirmed cases were then reviewed for Well's clinical probability scoring. All quantitative serum D-dimer measurements were performed using Latex agglutination tubidimetric immunoassay method with a fully automated coagulation analyzer. The diagnostic radiological investigations were Computed Tomography Pulmonary Angiography (CTPA), V/Q scans and Ultrasound Doppler studies. *Results.* 434 D-dimer values were included in the study. 371 were being investigated for pulmonary embolism whilst 63 were for deep venous thrombosis. Seven patients were radiologically proven to have pulmonary embolism i.e. negative predictive value of 98.12%, 95% confidence interval [CI], 96.1-99.2%. Four positive D-dimer values had positive radiological studies for DVT i.e. negative predictive value of 98.95% ,95% confidence interval [CI], 84.5% to 98.2%. Hence, the overall negative predictive value for thromboembolic events for the studied range of D-dimer values is 97.47% (95% confidence interval [CI], 95.5-98.7%. The well's clinical probability scoring for the patients with confirmed PE ranged from 3 to 7.5 (six patients with intermediate probability and one patient with high probability). *Conclusion.* The results of this study confirms that a d-dimer value <500 ng/mL used as a first diagnostic step in ruling out the diagnoses of pulmonary embolism and deep venous thromboses is a safe clinical practice when the pre-test clinical probability is low or intermediate. In patients with low clinical probability for thromboembolism and a D-dimer value of less than 500ng/mL the merit for further diagnostic radiological investigation remains to be seen.

0694

CHANGES IN PLASMA LEVELS OF ADAMTS 13 IN PATIENTS WITH VENOUS THROMBOEMBOLISM

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Background. Venous thromboembolism is a significant reason of morbidity and mortality. Hereditary and acquired risk factors are accused. However, in the studies being made for its etiology, number of patients with unidentified reason is substantially high. ADAMTS 13 (a disintegrin and metalloprotease with thrombospondin type 1 repeats) is a metalloprotease that splits von Willebrand factor (vWF) multimers in the plasma. Existence of large vWF multimers in plasma due to absence of ADAMTS 13 is the major factor in the pathogenesis of thrombotic thrombocytopenic purpura. The absence of ADAMTS 13 is also shown in also certain diseases where there is increased tendency to thrombosis (ischemic stroke, ischemic hearth disease etc.). *Aims.* The aim of this study is to determine the relation between thrombosis and ADAMTS 13, vWF antigen levels those detected in the plasma of patients with venous thromboembolism. *Methods.* Thirty patients with venous thrombosis and pulmonary embolia with 30 healthy individuals of the same age and sex were participated in our study. Diabetic, icteric patients, patients whose serum samples were lipemic, who had physical or surgical trauma, who have used drugs that influence thrombocyte functions, patients with acute coronary syndrome, kidney failure, liver disease, malignancy, collagenous tissue diseases and pregnants were excluded from our study. The plasma levels of ADAMTS 13 and vWF antigen of the patients and control group were determined quantitatively by ELISA. *Results.* The median value for ADAMTS 13 in patient group was 280 ng/mL (min-max: 70-1120 ng/mL) and 665 ng/mL (min-max: 350-2500 ng/mL) in control group (reference: 610-850 ng/mL). This result was found statistically significant (P<0.0001). The median value for vWF antigen in patient group was 1728±616 mu/mL and this was also found statistically significant (P<0.001) when compared with the control group whose median value was 1037±496 mu/mL (reference: 100-1000 mu/mL). Summary/*Conclusions.* Significantly lower levels of ADAMTS 13 and significantly higher levels of vWF antigen suggest that these changes can be the result of this pathogenic process, instead of being an etiological factor in venous thromboembolism. If the levels of ADAMTS 13 and vWF antigen are normal after the treatment of thromboembolism, this will make our theory stronger.

0695

THE IMPACT OF THE G455A POLYMORPHISM ON FIBRINOGEN B-CHAIN GENE ON THE COAGULATION CASCADE IN PATIENTS WITH ESTABLISHED CORONARY ARTERY DISEASE

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Background. The G455A polymorphism on fibrinogen b-chain gene has been associated with increased fibrinogen levels in healthy individuals. However, the impact of this polymorphism on coagulation markers such as factor V (fV), factor X (fX) and thrombin remains unknown. In the present study we examined the impact of this polymorphism on the aforementioned markers in patients with stable coronary artery disease (CAD). *Methods.* The study population consisted of 398 subjects, 253 of which angiographically documented for CAD. The G455A polymorphism was detected by polymerase chain reaction (PCR) and appropriate restriction enzymes. Factor X, f(V) and thrombin time were measured by standard coagulometry techniques. *Results.* The genotype distribution was GG: 51.4%, GA: 37.9% and AA: 10.7% for patients with CAD, while GG: 54.5%, GA: 35.8% and AA: 9.7% for controls. No significant difference was observed in thrombin time (sec) for CAD patients *vs.* controls (18.7±1.5 *vs.* 18.5±1.7, P=NS), while this difference remained for 455G carriers *vs.* 455AA homozygotes in CAD (18.8±3.1 *vs.* 19.8±12.0, P=NS) and controls (19.6±1.9 *vs.* 18.8±2.1, P=NS). In addition, fV (%) was significantly higher in CAD patients than controls (121.7±28.4 *vs.* 108.01±23.7, P=0.0011), while no difference was observed for 455G carriers *vs.* 455AA homozygotes both in CAD patients (122.2±29.5 *vs.* 131.55±28.6, P=NS) and controls (107.61±23.2 *vs.* 111.4±24.7, P=NS). No significant difference was observed in fX (%) for CAD *vs.* controls (94.0±35.4 *vs.* 91.9±14.2, P=NS) and 455G carriers *vs.* 455AA homozygotes in controls (92.2±14.2 *vs.* 94.6±11.8, P=NS). Interestingly, in CAD patients 455AA homozygotes had significantly higher fX compared to 455G carriers (103.6±21.1 *vs.* 89.3±23.8, P<0.05). *Conclusions.* Our findings suggest that the G455A polymorphism on fibrinogen b-chain gene affects plasma levels of factor X in patients with stable coronary artery disease. These findings provide new insights in the associations between genetic variability of fibrinogen genes and thrombotic state.

0696

THE ROLE OF THE G455A POLYMORPHISM ON FIBRINOGEN B-CHAIN GENE IN ADVANCED ATHEROSCLEROSIS: EFFECTS ON FIBRINOGEN, D-DIMERS AND PLASMINOGEN LEVELS

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Background. Genetic polymorphism G455A on fibrinogen b-chain gene has been associated with increased fibrinogen levels in healthy individuals. However, the impact of this polymorphism on the coagulation cascade in patients with coronary artery disease (CAD) is unknown. In the present study we examined the impact of this polymorphism on fibrinogen levels, D-dimers levels and plasminogen levels. *Methods.* The study population consisted of 398 subjects, 253 of which angiographically documented for CAD. The G455A polymorphism was detected by polymerase chain reaction (PCR) and appropriate restriction enzymes. Fibrinogen levels were measured by immunonephelometry, while plasminogen and D-dimers levels were measured by standard coagulometry techniques. *Results.* The genotype distribution was GG: 51.4%, GA: 37.9% and AA: 10.7% for patients with CAD, while GG: 54.5%, GA: 35.8% and AA: 9.7% for controls. Patients with CAD had significantly higher fibrinogen levels (mg/dL) than controls (434.7±132.7 *vs.* 384.7±103.7, P=0.0002). In addition, in patients with CAD fibrinogen levels were importantly higher in the 455AA homozygotes *vs.* 455G carriers (561.6±127.3 *vs.* 442.6±132.2, P<0.0001), while no similar difference occurred in controls (420.9±143.9 AA *vs.* 380.6±98.5 GG+GA, P=NS). Moreover, D-dimers levels (mg/L) were significantly higher in CAD patients than controls (415.02±201.9 *vs.* 332.8±199.4, P<0.0001). However, no significant difference was observed for 455G carriers *vs.* 455AA homozygotes for both CAD patients (394.8±189.6 *vs.* 489.6±318.4, P=NS) and controls (368.1±286.9 *vs.* 406.9±258.2, P=NS). Finally, CAD patients and controls had no significant difference in plasminogen levels (u/mL) (119.2±79.7 *vs.* 113.9±22.9, P=NS). Although, CAD 455AA homozygotes had higher plasminogen levels compared to 455G carriers (116.9±10.6 *vs.* 109.5±17.2, P=NS) this difference did not reach any statistical significance, while similar results were observed in controls for 455AA homozygotes *vs.* 455G carriers (116.7±15.9 *vs.* 113.6±23.7, P=NS). *Conclusions.* Our findings showed that genetic polymorphism G455A on fibrinogen b-chain gene strongly affects fibrinogen levels. However, this polymorphism fails to affect D-dimers levels as well as plasminogen levels in patients with stable coronary artery disease.

0697

COMPOSITION AND SIGNIFICANCE OF SPLENIC GAMNA GANDY BODIES IN SICKLE CELL ANAEMIA

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Background. Children with sickle cell anaemia (SCA) may undergo acute splenic sequestration (ASS). Splenectomy (SP) is usually performed after two clinically significant ASS in an attempt to reduce the incidence of further events but its efficacy remains controversial. Histological studies of spleens have on occasion revealed the presence of granuloma-like nodules, known as Gamna-Gandy bodies (GGB). Moreover, amorphous inclusions have been seen in GGB, however their significance is unknown. *Aims.* To identify the nature and the significance of GGB in SCA. *Methods.* The medical case records and histological-samples of consecutive SCA children treated with open SP between 2001-2007 at Our Lady's Children's Hospital, Dublin were reviewed. Seventeen patients were identified, in all cases histological-samples were made available. Crises pre- and post-SP were recorded. GGB were also studied by scanning electron microscopy (SEM) and X ray fluorescence in energy dispersive spectroscopy mode (XRF-EDS). An extensive

literature search on GGB was performed. Original articles from the early studies (1905) in several languages were provided through a library specialising in antique medical articles. Results. GGB were identified in 7 (41%) of our patients, amorphous inclusions of different sizes were always seen in GGB. Patients age correlated significantly with GGB presence (P=0.0025). Chest crisis were strongly associated with GBB before and after SP (P=0.00). SEM analysis on high magnification demonstrated the crystalline nature of GGB inclusions and the chemical composition: carbon (C) 47.1%; oxygen (O2) 29.7%; phosphorus (P) 9.0%; potassium (K^+) 0.4%; calcium (Ca2+) 6.4%; iron (Fe^{2+}) 7.4%. Using SEM at low-magnification, a crystal-formation gradient was observed, arising from the red pulp (RP) to the white pulp (WP), with maximal expression in the WP in GGB proximity. The early pathological descriptions of GGB were reviewed together with the new spectrographic finding. *Summary/Conclusions.* This study demonstrates that GGB-crystals formations in SCA are derived from RCC breakdown when periarterial haemorrhage occurs and will be less expressed in the RP, in contrast with delicate sinusoidal haemosiderin, which is a result of chronic haemolysis. We also demonstrated that GGB presence is age-dependent, in keeping with the first morphological studies of the beginning of the century. Moreover a strong relationship between the presence of GGB and chest crises pre or post SP was seen. Recent studies shown that post SP, patients on hydroxyurea (HU) are less likely to develop further crises. Taken together these considerations suggest that post SP, particularly when GGB are seen, patients may benefit from prophylactic HU treatment. Larger studies are now warranted to support these findings.

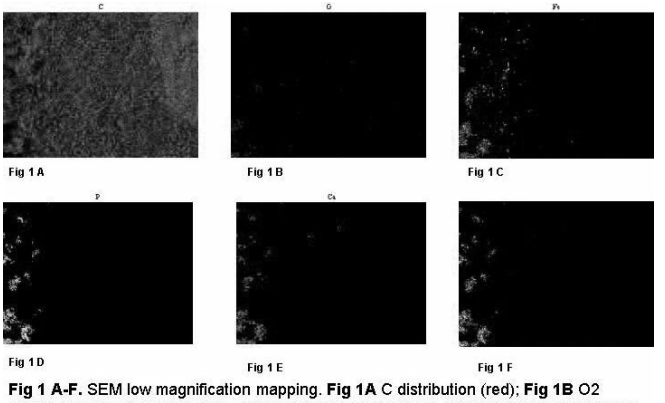

Fig 1 A-F. SEM low magnification mapping. **Fig 1A** C distribution (red); **Fig 1B** O2 distribution (blue); **Fig 1C** Fe2+ distribution (pink); **Fig 1D** P distribution (green); **Fig 1E** Ca2+ distribution (red); **Fig 1F** overlapping fields showing that C,Ca2+, Fe2+, and P are all crystals component.

Figure 1.

0698

THE PROTEIN C ASN2ILE SUBSTITUTION CAUSES MARKED DISCREPANCY BETWEEN PROTEIN C ACTIVITY CLOTTING ASSAYS

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Background. Laboratory measurement of protein C (PC) may be required to detect PC deficiency in individuals with premature or familial thrombosis and infants with purpura fulminans. Commercially available assays include ELISA PC antigen assays and PC activity assays in which snake-venom extracts are used to first generate activated PC (APC) in test plasma. APC activity is then measured using chromogenic or clotting end-point assays. Current consensus guidelines recommend that both chromogenic and clotting assays are performed to identify all functional PC variants. We have previously reported a kindred in which co-inheritance of the independent PROC mutations c.669C>A (predictive of Ser181Arg) and c.131C>T (predictive of Asn2Ile) caused a severe PC deficiency phenotype. The Ser181Arg PC variant was associated with a simple quantitative (type I) PC deficiency. However, PC with the Asn2Ile substitution in the PC Gla domain ω loop was dysfunctional and in heterologous cells, disrupted APC/PC binding to the endothelial protein C receptor and phosphoipid (PL). *Aims.* To characterise the Asn2Ile PC variant using commercially available PC assays. *Methods.* We obtained venous blood samples from the following family members with informed consent; II-3 (asymptomatic heterozygote for PC Ser181Arg); II-4 (asymptomatic heterozygote for PC Asn2Ile) and III-1 (purpura fulminans and compound heterozygous for PC Asn2Ile + Ser181Arg). PC antigen was measured using ELISA. PC activity was measured using 3 chromogenic assays (Berichrom Protein C, Stachrom Protein C and an in-house method using Protac® PC activator and the S2366 chromogenic substrate) and 3 clotting assays (Staclot Protein C, ProClot and CRYOcheck Clot C). Results. II-3 (Ser181Arg) showed concordant reductions of PC with the PC antigen assay and PC activity measured using chromogenic and clotting assays confirming a type I defect (Table). II-4 (Asn2Ile) showed normal PC antigen and PC activity with the chromogenic assays. PC activity was also normal with the ProClot clotting assay and close to the lower limit of the laboratory reference range with the CRYOcheck Clot C clotting assay. However, PC activity was clearly reduced using the Staclot Protein C clotting assay (Table) revealing a partial type IIb PC deficiency. III-1 (Ser181Arg+Asn2Ile) showed a partial reduction in PC antigen and PC activity measured using the chromogenic, ProClot and CRYOcheck Clot C clotting assays, suggestive of a partial type I PC deficiency. However, PC activity measured with the Staclot Protein C clotting assay was markedly reduced in keeping with the severe PC deficiency phenotype (Table). Summary and Conclusions. In PC activity clotting endpoint assays, APC is required to bind PL to exert an anticoagulant effect. Therefore, reduced PL binding caused by the Asn2Ile PC substitution is expected to manifest as reduced PC activity. Differences in PL concentration or duration of incubation with APC in the different clotting assays may account for the differences we have observed in assay performance. These data illustrate that a wider range of PC assays may be necessary to identify all functional PC variants.

Table 1. PC assay results for the study kindred.

		II.3	II.4	III.1	ref range (u/dL)
Antigen	ELISA*	48	95	42	70 – 140
Chromogenic	Berichrom Protein C	48	104	40	70 – 140
	Stachrom Protein C	50	98	62	70 – 130
	In-house assay**	50	86	58	79 – 161
Clotting	Staclot Protein C	56	48	9	70 - 130
	ProClot	65	87	43	70 – 140
	CRYO*check* Clot C	-	72	49	73 – 155

* Polyclonal rabbit anti-human PC antibody
** Protac® PC activator and S2366 chromogenic substrate

0699

CAN VERY HIGH VALUE OF D-DIMER PREDICT THE PRESENCE OF THROMBOEMBOLIC DISORDERS?

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Background. D-dimer quantitative test is mainly used to rule out the presence of thromboembolic diseases (TED) when the result is below the cutoff point. *Aims.* To see if very high D-dimer value (100 times above the cutoff point) can exclusively indicate the presence of TED. *Methods.* D-dimer was detected by the method of quantitative immunoturbidimetric assay (Innovance* D-Dimer, Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany). The normal value is 0.2-0.7 mg/L FEU (Fibrinogen Equivalent Units). The cutoff point to rule out TED is set at 0.5 mg/L FEU or less. During the year of 2009, 1053 D-dimer tests were performed in our hospital. We analyzed the results of these 1053 samples, mainly to find out the causes of very high D-dimer. *Results.* The mean value of D-dimer in the 1053 tests was 8.56 (SD 30.87) mg/L FEU, with range from <0.19 to 563.2 mg/L FEU and the medium was 2.1 mg/L FEU. Of them, 28 samples (2.7% of total tests) from 21 patients were found to have very high D-dimer value: >50 mg/L FEU, ranging from 51.0 to 563.2 mg/L FEU. We analyzed these 21 patients to see the underlying diseases which causing the very high D-dimer. Of the 21 patients, 9 (43%) were proved to have TED, 1 had suspected TED, but not proved by CT angiogram, 3 had massive gastrointestinal (GI) or abdominal bleeding, 2 patients had cardiac arrest with samples taken immediately after recovery from cardiopulmonary resuscitation (CPR), 1 had postpartum HEELP syndrome, 1 had hyperfibrinolysis, 1 had ALL on chemotherapy, 1 had multiple traumatic injury caused by traffic accident, 1 was after thrombolytic therapy, and 1 had sickle cell disease with acute chest syndrome with sample taken just before the patient's death. *Conclusions.* Although TED was the most

frequently seen disorders in patients with very high D-dimer value, very high D-dimer was not necessary exclusively the marker of TED. Other disorders such as massive GI or abdominal bleeding, status post CPR, multiple traumatic injuries, hyperfibrinolysis and HEELP syndrome can also have very high D-dimer.

0700

SUCCESSFUL REMOVAL OF DABIGATRAN IN FLOWING BLOOD WITH AN ACTIVATED CHARCOAL HEMOPERFUSION COLUMN IN AN IN VITRO TEST SYSTEM

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Dabigatran etexilate is an oral direct thrombin inhibitor approved for prevention of VTE in patients after orthopedic surgery, and has shown efficacy and safety in prevention of stroke in patients with atrial fibrillation and in VTE treatment. A general limitation of anticoagulant use, particularly with chronic treatment, is the lack of a rapidly acting antidote to reverse its effects in emergency situations of overdose or uncontrolled bleeding. Dabigatran has a low plasma protein binding and thus can be dialysed. In addition, it could potentially be removed from patient blood by charcoal hemoperfusion. This was tested *in vitro* using the Adsorba™ cartridge, which contains specially activated carbon that allows hemoperfusion treatment and is clinically available for use in drug and toxin intoxifications. Dabigatran was added to 5L bovine whole blood (anticoagulated with sodium citrate) to achieve supratherapeutic concentrations (1000 ng/mL) and was pumped through a tubing system (flow rate increased every 2 hrs, between 150-350 mL/min) across a Gambro Adsorba™ cartridge (n=3 experiments). Blood pre and post Adsorba™cartridge was serially sampled at 15-30 min intervals over 6 hours. Plasma was obtained by centrifugation and frozen. Dabigatran was measured using standard LC-MS/MS methods. To test the effect of dabigatran on clotting in bovine blood an *in vitro* standard curve was also performed using routine clotting tests, thrombin time, activated partial thromboplastin time and ecarin clotting time. Initial circulating dabigatran levels in bovine blood were 1140±40 ng/mL. As blood was pumped across the Adsorba cartridge, circulating dabigatran was almost completely removed, i.e. at 15 min dabigatran pre filter levels were 710 ng/mL, this was reduced 16-fold to 42 ng/mL post filter (Fig1). Accordingly, dabigatran plasma levels in the 5 L volume decreased exponentially resulting in a ~85 % reduction when 1.8 plasma volumes were treated after 60 min and a more than 95% reduction when 3.6 plasma volumes were treated after 120 min. Dabigatran also prolonged clotting in bovine blood in a concentration-dependent manner. These data demonstrate that dabigatran can be removed from whole blood *in vitro*, by adsorption across an activated charcoal column (Adsorba™ Cartridge). Thus this may be a potential method to remove dabigatran rapidly in blood in emergency situations; however, further testing is required *in vivo* before this can be recommended for the clinic.

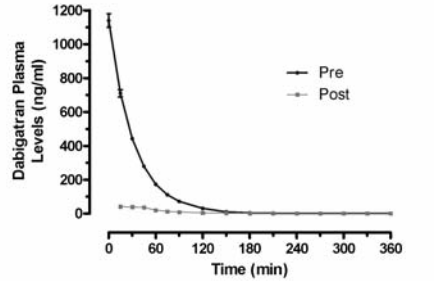

Figure 1. Mean +/- SEM, n=3 experiments.

0701

NATIONAL REGISTRY OF THROMBOPHILIC STATES IN SLOVAK REPUBLIC

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Background. Thrombophilic states (TS) are inherited or acquired hemostatic disorders associated with increased risk of thrombosis. Their most important clinical manifestation is venous thromboembolism (VTE). *Aims.* Primary goal of our project was to find out prevalence of the most frequent inherited TS in families of patients with thrombosis in Slovakia using National Registry of Thrombophilia States (NRTS). *Methods.* Patients from all regions of Slovakia with history of thrombosis and their relatives were examined for TS including FV Leiden and prothrombin mutation, protein C (PC), protein S (PS) and antithrombin (AT) defects, sticky platelets syndrome (SPS), hyperhomocysteinemia, etc. All patients were informed and agreed to participate at the database of NRTS. *Results.* There was confirmed the presence of TS in 2912 subjects of NRTS. The most frequent TS was FV Leiden mutation with prevalence of 42%, PS defect was found in 13%, prothrombin G20210A mutation in 10%, SPS in 9%, PC and AT defects in 5%. VTE was proved in 52% of TS (45% venous thrombosis and 7% combination of VT and pulmonary embolism), arterial thrombosis was present in 5% and other complication in 3% of patients. 600 patients with unexplained cause of thrombosis were examined for SPS, which was confirmed in 150 patients (25%). Summary/*Conclusions.* Our results from NRTS suggest that TS are common causes of thrombosis in Slovak population. The most frequent TS in families of patients with thrombosis are FV Leiden mutation (42%) and PS defect (13%). Interesting finding seems high prevalence of SPS in patients with unexlained thrombosis (25%). This work was supported by grants VEGA 1/0067/08, 1/0018/10 and by the European Regional Development Fund (ERDF) Project "Center of Excellence for Perinatological Research".

0702

THROMBIN GENERATION POTENTIAL OF ACUTE PROMYELOCYTIC LEUKEMIA (APL) CELLS TREATED WITH ARSENIC TRIOXIDE (ATO) AND ALL-TRANS RETINOIC ACID (ATRA)

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Background. Therapeutic approaches based on ATRA in combination with ATO are effective in curing APL and reuce chemotherapy-associated long-term complications in these patients. In addition, ATRA and ATO improve the APL life-threatening coagulopathy and affect APL cell procoagulant activity. *Aims.* In this study we evaluated, for the first time, the effect of ATRA and ATO, and of their combination, on the global APL cell (NB4) hemostatic potential as evaluated by the calibrated automated thrombogram (CAT). *Methods.* NB4 cells were incubated with either 0.1 μM ATO, 1 μM ATRA, their combination, or the vehicle (control). After 24h incubation, the NB4-TG potential was evaluated by the CAT assay in both normal pooled plasma (NPP) and factor VII-deficient plasma (FVII-DP). In parallel the levels of procoagulants [tissue factor (TF) and cancer procoagulant (CP)], anticoagulant (thrombomodulin, TM) and markers of differentiation, proliferation, apoptosis/necrosis were evaluated. *Results.* ATRA and ATRA/ATO treatment significantly reduce the TG of NB4 cells in both NPP and FVII-DP, whereas ATO only gives a slight reduction. TG potential of NB4 cells was significantly lower in FVII-DP than in NPP, confirming a major role for TF-like procoagulant activity. The residual TG activity in FVII-DP stands for the presence of FVII-independent procoagulants (i.e. CP, phospholipids). While all the treatments determined a decrease in TF and CP levels, and mainly in ATRA-treated cells, a parallel increase of TM was observed in ATRA or ATRA/ATO treated cells. Significant correlations were found between the different TG parameters and the levels of TF and CP, the rate of cell proliferation and differentiation. Summary/*Conclusions.* The pharmacological modulation of APL cell hemostatic potential by ATRA and ATO can be characterized by the CAT assay . It might possibly help to define the role *in vivo* of different TG phenotypes in the outcome of the APL-associated coagulopathy and in the patient prognosis.

0703

PROTHROMBIN ACTIVITY AND ANTIGEN IN CARRIERS OF PROTHROMBIN G20210A MUTATION

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Background. Patients with prothrombin G20210A mutation have higher plasma prothrombin levels than subjects with a normal genotype.The relationship of the prothrombin (Factor II) activity (FII:C) with the related antigen (FII:Ag) is poorly understood and it is unclear if the high levels of FII:Ag are correlated with high FII:C levels in subjects with

G20210A mutation. *Aims.* Aim of our study is the evaluation of the FII:C, FII:Ag and prothrombin fragments 1+2 (F1+2) in patients with prothrombin G20210A mutation as compared to a normal population. *Methods.* Subjects of our study were patients with a personal or family history of venous/arterial thromboembolic events and with G20210A mutation. Subjects without personal or family history of venous/arterial thromboembolic events and without G20210A mutation, matched for age and sex, were the control population. In patients and controls, the presence of all other markers of congenital and acquired thrombophilia were excluded. Normal ranges of FII:C and of FII:Ag were 60 -120 U/dl, and 50-150 U/dl, respectively. Normal range of F1+2 was 0.4 - 1.1 nmol/L. *Results.* We studied 63 subjects with prothrombin G20210A mutation, all cases were in the heterozygous status (Group A). Sixty-three healthy subjects without the mutation, were the control group (Group B). In Group A, the mean value of FII:C was 122.6 U/dL (SD = 18.90), the mean value of FII:Ag was 141.8 U/dL (SD= 48.20) and of F1+2 was 0.55 nmol/L (SD=0.11). In Group B the mean value of FII: C was 105.7 U/dL (SD = 12), the mean value of FII: Ag 98.6 U/dL (SD= 22) and of F1+2 was 0.59 nmol/L (SD=0.14). In Group A, the mean ratio between values of activity and antigen was 0.92 (SD=0.20) and in Group B it was 1.11 (SD=0.20). In Group A 34/63 (54%) patients showed almost one previous thromboembolic event; we compared patients with and without previous thromboembolic events: mean values of FII:C were 124.9 U/dL (SD=17) and 119.8 U/dL (SD=20.80), respectively and mean values of FII:Ag were 142.2 U/dL (SD=50.90) and 141.4 U/dL (SD=45.70), respectively; mean values of F1+2 were 0.56 nmol/L (SD=0.11), and 0.54 nmol/L (SD=0.10), respectively. Mean ratios between activity and antigen in patients with and without thrombotic events were 0.95 (SD=0.22) and 0.89 (SD=0.17), respectively. *Conclusions.* In comparison with Group B, subjects of Group A showed either higher levels of FII:Ag (P<0.000) and of FII:C (P=0<000), or a lower ratio between FII:C and FII:Ag (P<0.000). In patients of Group A with and without previous thromboembolic events, no differences concerning levels of FII:C (P=0.720), FIIAg (P=0.664), and F1+2 (P=0.097) were recorded. These data suggest that the G20210A mutation induces a procoagulant effect, but high levels of FII:Ag seem to play a greater role in comparison to the activity. In G20210A mutation carriers, we did not find any correlation between the levels of FII:C and of FII:Ag with the occurrence of thrombotic events.

0703a

GENDER BIAS OF CARDIOVASCULAR RISK IN CAUCASIAN TYPE II DIABETICS: A HAEMOSTATIC EXPLANATION?

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Background. Cardiovascular disease (CVD) is the largest single cause of death in patients with diabetes, particularly in females.[1] The excess cardiovascular morbidity and mortality among female diabetics in developed countries has not been fully explained by conventional risk factors such as smoking, hypertension and high serum cholesterol.[2] Prospective studies in the general population have identified various haemostatic markers for the subsequent development or progression of large vessel atherosclerosis.[3,4] Evidence of increased thrombotic tendency through haemostatic parameters in a gender-specific fashion could have aetiological, preventive, and therapeutic implications in type II diabetics. *Aims.* This study examines specific parameters of the coagulation and fibrinolytic system in Irish Caucasian type II diabetics to examine their possible contribution to the increased CVD risk in diabetic females. *Methods.* A sample of 113 Irish caucasian (60 male and 53 female) type II diabetics attending the Mid-Western Regional Hospital Limerick submitted signed consent according to the protocol approved by the ethics research committee. Body Mass Index (kg/m^2), blood pressure, blood phenotype, current medications, evidence of positive family history of diabetes and any previous cardiovascular event(s) were recorded. Clinical CVD was defined as coronary heart disease, stroke, or peripheral vascular disease. Haemostatic parameters examined included Factor VII (commercial deficient FVII plasma), Factor VIII (commercial deficient FVIII plasma), von Willebrand factor (immunoturbidometric method), fibrinogen (Clauss), Thrombin Activatible Fibrinolytic Inhibitor (chromogenic method) and Plasminogen Activator Inhibitor -1 antigen (ELISA). Statistical analysis was performed using SPSS Version 16.0. A value of P<0.05 was considered to indicate a statistically significant difference. Haemostatic variables found to be significantly different between the genders were adjusted using multivariate analysis (ANOVA) for age, BMI, smoking habit, HbA1c, cholesterol, clinical CVD, hypertension, and family history of diabetes. Results. The only significant difference observed in clinical and biochemical variables was that more males were current smokers (P=0.001). Female diabetics had significantly higher unadjusted FVII levels (P=0.011) than their male counterparts (Table 1). However, following adjustment for clinical and biochemical variables, FVII was no longer significantly different between genders but significantly different between cholesterol levels (P=0.007), smoking (P=0.039) and clinical CVD (P=0.035) plus interactions between the smoking/family history of diabetes groups (P=0.043) and smoking/CVD/family history of diabetes groups (P=0.023). Unadjusted fibrinogen levels were significantly higher in females (P=0.001). However, this difference in fibrinogen according to gender was corrected without finding a single significant determinant. Although this study found significantly higher unadjusted TAFI levels in females than males (P=0.022), this difference was significantly related to total cholesterol levels (P=0.046) and was not predicted by gender. Conclusions. As the prevalence of type II diabetes continues to increase worldwide, there is an enhanced need for effective disease management. This study identifies specific traditional cardiovascular risk factors in females which may be associated with atherosclerosis through modifications of the plasma levels of certain haemostatic factors (FVII, fibrinogen and TAFI) and that haemostatic parameters do not independently contribute to the gender bias in CVD risk. Our findings provide additional support for control of lifestyle risk factors for cardiovascular health in female diabetics.

Table 1. Gender comparison of haemostatic parameters.

Parameter	Males (n=60)	Females (n=53)	*P*
FVII, %	106.8 [25.9]	118.9 [23.6]	0.011
FVIII, %	189 [141-228.5]	170 [132-214]	NS
vWF, %	136.5 [46.8]	134.9 [46.2]	NS
Fibrinogen, g/l	3.54 [0.67]	4.01 [0.76]	0.001
PAI-1 antigen, ng/ml	46.6 [13.3]	45.3 [13.9]	NS
TAFI, %	109.8 [22.5]	119.3 [20.9]	0.022

HbA1c, total cholesterol, FVIII reported as median with interquartile range. FVII, vWF, Fibrinogen, PAI-1 and TAFI reported as mean ± standard deviation. FVII differences between genders dependent on cholesterol levels (*P*=0.007), smoking (*P*=0.039), clinical CVD (*P*=0.035), smoking/family history of diabetes groups (*P*=0.043) and smoking/CVD/family history of diabetes groups (*P*=0.023). The fibrinogen difference had no single significant determinant. The TAFI difference was significantly related to total cholesterol levels (*P*=0.046) and not predicted by gender.

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Bleeding biology

0704

BIOCHEMICAL MARKERS OF BONE TURNOVER USED TO EVALUATE AND COMPARE DUAL ENERGY X-RAY ABSORPTIOMETRY (DXA) AND QUANTITATIVE ULTRASONOGRAPHY (QUS) IN BOYS WITH HAEMOPHILIA

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Background. Recent studies indicate a high prevalence of impaired bone properties in adult patients with haemophilia. Even among boys, decreased BMD values have been constantly reported and attributed to a multifactorial pathogenesis that mainly includes immobilization and increased bone turnover mediated by the secretion of pre-inflammatory cytokines due to haemophilic arthropathy. *Aims.* The aim of this study was to assess biochemical markers of bone turnover in boys with haemophilia, to investigate the pathogenesis of impaired bone quality and to compare two densitometric methods [Dual energy X-ray Absorptiometry (DXA) and Quantitative UltraSonography (QUS)]. *Methods.* Twenty-six boys with a mean decimal age of 12.08±4.44 years were included in the study. In every patient and in 13 controls serum levels of soluble receptor activator of nuclear factor κB ligand (sRANK-L), osteoprotegerin (OPG) and osteocalcin (OC) were measured. Additionally, all patients had a DXA scan performed at lumbar spine and were evaluated with QUS at radius and at tibia. Finally, joint evaluation was performed using the Hemophilia Joint Health Score (HJHS). *Results.* Boys with haemophilia had significantly higher serum levels of sRANK-L (21.04±4.78 *vs.* 18.58±2.28 ng/mL, P=0.038) and of OC (5.35±2.29 *vs.* 3.09±0.61 ng/mL, P=0.002) and significantly decreased levels of OPG (15.78±2.53 *vs.* 23.79±4.39 pg/mL, P<0.001) compared to controls. Only 2 out of 26 patients (7.7%) had Bone Mineral Density (BMD) Z-scores <-2, whereas another 4 patients (15.4%) had BMD Z-scores between -1 and -2. QUS values in both radius and tibia were generally within the normal limits as only one patient had radius and other two had tibia QUS Z-scores <-2. No agreement was recorded between QUS and DXA in identifying patients at risk for osteoporosis (k = 0.275, P=0.063) and no correlations were observed between lumbar BMD and QUS measured either at radius or tibia. QUS Z-scores at tibia were significantly correlated to HJH Scores (r=-0.450, P=0.040), whereas lumbar BMD Z-scores were significantly correlated to BMI Z-scores (r=0.500, P=0.009). Finally, multivariate analyses showed that levels of sRANK-L and OC are the most important predictors of SOS values at tibia, whereas for lumbar BMD values BMI values has the highest predictive value. Symmary/*Conclusions.* An increased osteoclastic activity followed by a compensatory osteoblastic function was indicated by biochemical markers of bone turnover, although no impaired bone properties, measured either by DXA or QUS were recorded in boys with haemophilia. No agreement was recorded between the two densitometric methods, whereas biochemical markers and HJH Score seemed to correlate more reasonably to QUS parameters measured at tibia.

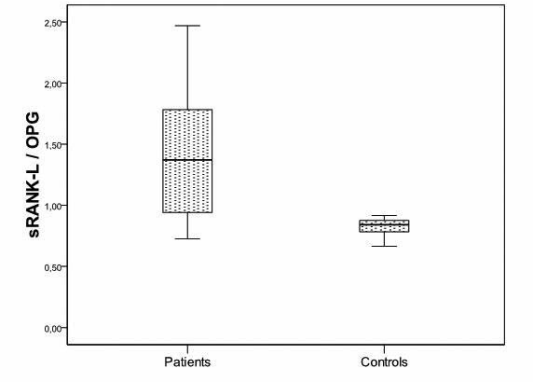

Figure 1. Significantly higher sRANK-L/OPG ratio in patients.

0705

MOLECULAR CHARACTERIZATION OF AN ITALIAN COHORT OF 10 PATIENTS WITH TYPE 3 VON WILLEBRAND DISEASE: EXPRESSION STUDIES OF 3 MISSENSE MUTATIONS

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Background. Type 3 von Willebrand disease (VWD3) is a severe autosomal recessive inherited bleeding disorder caused by a virtually complete absence of von Willebrand Factor (VWF). Usually patients are homozygous or compound heterozygous for null alleles due to nonsense mutations, small insertions/deletions, splice site defects and, more rarely, large gene deletions. Nevertheless, several missense mutations have been reported. *Aims.* To better understand the molecular defects of VWD3, we have genetically analyzed 10 patients previously diagnosed with VWD3. *Methods.* Molecular defects of VWF were evaluated by DNA direct sequencing, High Resolution Melting (HRM) analysis and duplex PCR. Moreover, we performed *in vitro* expression studies of the missense mutations, to confirm their link with the disease. Mutant and wild-type (WT) expression vectors were used for transient transfection, alone and together (hybrids), in COS-7 cells. Conditioned media and cell lysates were collected and recombinant VWFs (rVWFs) were quantified by VWF: Ag and evaluated for multimeric structure. *Results.* Twenty-six exons were analyzed by direct sequencing whereas, the remaining twenty-five were evaluated by both HRM and sequencing analysis. Direct sequencing (see table) revealed eleven mutations (shown in italics); HRM analysis detected two defects (shown in bold) and one large deletion was identified by duplex PCR. Nine out of thirteen identified mutations were novel (*), which are 5% of VWD3 previously reported mutations. Four patients were found to carry mutations in the homozygous state, but only two of them were found to be born from a consanguineous marriage. The large deletion involving exons 1-3, recently reported as the most frequent in the Hungarian population, was found in two patients. Expression studies of missense mutations (C2184S, C2212R and C2325S) showed a strongly reduced secretion of mutant proteins, with only dimers being visualized using multimer analysis. Hybrids rVWFs showed mildly reduced secretion and a full set of multimers. Higher VWF: Ag levels were detected in cell lysates of mutants and hybrids rVWFs in comparison to the WT. *Conclusions.* HRM analysis, used for the first time in the molecular diagnosis of VWD3, shows a reduced ability in detecting mutations on VWF. The presence of many polymorphic sites in the VWF coding region has, so far, limited the use of this technique to twenty-five exons of the gene only. The three detected missense mutations were confirmed to be disease related by expression studies. All of them involve a cysteine residue, important in the correct folding/processing/secretion of the neo-synthesized VWF. Further analysis should be performed on the two partially characterized patients to evaluate intronic mutations or heterozygous large deletions, whereas, mRNA analysis should be carried out to confirm the candidate splice site mutations identified.

Table 1.

PZ	MUTATION	MUTATION EFFECT	EXON INTRON INVOLVED
1	**2157delA** / *7729+7C>T*	**D720TfsX21** / *Splice site*	16 / IVS45
2	*6651G>C* / ?*	*C2184S* / ?*	37 / ?
3	*4576C>T* / **6973T>A****	*Q1526X* / **C2325S****	28 / 40
4	*6634T>C* / 6634T>C**	*C2212R* / C2212R**	38 / 38
5	del ex 1-3 / *3940delG**	Large deletion / *V1314SfsX33**	1,2,3 / 28
6	*8155+1G>T* / 8155+1G>T**	*Splice site* / Splice site**	IVS50 / IVS50
7	*4645G>T* / 7344C>A**	*E1549X* / C2448X**	28 / 43
8	*2269delCT / 2269delCT*	*L757VfsX22 / L757VfsX22*	17 / 17
9	*658-2 A>G* / 658-2 A>G**	*Splice site* / Splice site**	IVS6 / IVS6
10	del ex 1-3 / ?	Large deletion / ?	1,2,3 / ?

0706

HLA GENOTYPING IN CHILDREN WITH SEVERE HAEMOPHILIA A AND RELATION TO INHIBITORS DEVELOPMENT

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Background. Alloantibodies (inhibitors) against FVIII in severely haemophilia A (HA) affected patients (20-30%) represent a major complication of treatment, affecting patient's care, by rendering FVIII substitution therapy ineffective. Inhibitors development seems to be multifactorial and is based on complex immunological factors. Genetic factors, such as the type of FVIII gene mutation and immune response genes, e.g major histocompability complex, influence the risk of inhibitor formation. *Aim/Materials/Methods.* To investigate a possible correlation between FVIII gene intron-22 inversion and HLA factors with the risk for inhibitors development, we performed Long Range PCR for detection of intron-22 inversion and PCR-SSP and PCR-SSO for genotyping of HLA A, B, Cw, DRB1 and DQB1 alleles in 38 children with severe HA, exclusively treated with recombinant products (PUPs). X2 test and Fischer's exact test were used for statistical analysis. *Results.* On the whole, 19 patients had developed inhibitors (Group I) 13 of them high responding, while 19 had not (Group II). There were no statistically increased intron-22 inversion frequencies in the inhibitor positive group: 17/38 (44.7%) children were found positive for intron-22 inversion and nine of them were positive for inhibitors (high titre: 7, low titre: 2). Comparing frequencies of HLA alleles between Group I and Group II, statistical significant differences were found in the following genotypes promoting inhibitors development: DRB1*0101(P=0.016) and DQB1*0501 (P=0.017, OR=10.5) while a trend revealed regarding alleles DRB1*01 (P=0.08), DRB1*1302 (P=0.071), and DQB1*0601 (P=0.075). On the other hand, the following HLA alleles seemed to confirm protection from inhibitors development: HLA-B*18 (P=0.056), DRB1*11 (P=0.009, OR=6.1), DQB1*03 (P=0.004, OR=7.8) and DQB1*0301 (P=0.009, OR=6.1). *Conclusion.* The HLA genotyping in our study shares in common findings with other studies in Caucasian haemophilic population. Nevertheless, some differences observed between the present and some previously reported HLA associations in HA patients with inhibitors could be attributed to the varying ethnic immunogenetic profile. The biological significance of HLA associations in clinical application has to be prospectively confirmed, in order to prevent inhibitor development by modifying treatment strategies.

0707

INTRA-ETHNIC AND FAMILY STUDIES OF THE INHERITANCE OF GENE MUTATIONS IN GLANZMANN THROMBASTHENIA USING AN EMERGING METHOD: HIGH RESOLUTION MELTING CURVE ANALYSIS

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Background. High Resolution Melting (HRM) curve analysis is a new, homogeneous, closed-tube, post-PCR method, permitting the identification of genetic variations in PCR amplicons. It uses a generic DNA fluorescent dye at high, saturating, concentration with homogeneous staining of dsDNA, without inhibiting the PCR reaction. Samples can be discriminated according to their sequence, length, GC content or strand complementarity. Our investigations have focused on Glanzmann thrombasthenia (GT), an autosomal recessive bleeding disorder caused by quantitative and/or qualitative deficiencies of the αIIbβ3 integrin encoded by the ITGA2B (30 exons) and ITGB3 (15 exons) genes. Classically, GT patients are homozygous or compound heterozygous for nonsense or missense mutations, small insertions or deletions and splice site defects that occur across both genes. Current screening for GT is expensive and slow; for most new families it requires sequencing of all exons and their splice sites. *Aims.* To test the relevance and the accuracy of HRM in genetic diagnosis for four known molecular variations of GT, in two different contexts: ethnic and familial inheritance. *Patients and Methods.* Studied were DNA samples from nine French families (25 subjects), including members of seven families from a gypsy ethnic group. Mutations screened, were the c.1544+1G>A transversion in intron 15 of ITGA2B, also called the 'Gypsy' mutation, leading to abnormal RNA splicing, a truncated protein and absent αIIbβ3 in platelets; a c.685T>C transition in exon 5 (ITGB3), leading to a Leu196Pro substitution and a functionally defective integrin; c.724C>T in exon 5 (ITGB3) giving a stop codon (Arg216X); and c.1871G>A in exon 11 (ITGB3) giving a Cys598Tyr substitution and a partially activated integrin. Conditions were established for HRM analysis on a ROCHE Light Cycler 480 apparatus, and all results were confirmed by direct sequencing. A control DNA sample was included in all experiments; it was also mixed with patient samples to reveal potential hetero-duplex complexes. *Results.* HRM analysis was successfully performed for all subjects. For families of gypsy origin, it identified 8 homozygous subjects, 5 heterozygous and 6 without the intron 15 c.1544+1G>A transversion on either chromosome. Heterozygosity for the c.685T>C transition in exon 5 of the ITGB3 gene previously detected in a patient with variant GT, was confirmed by HRM in all three members of his family. In contrast, a second c.1871G>A transition in exon 11, and was only present in the propositus who was therefore a compound heterozgote. This co-inheritance of qualitative inhibitory (β3 Leu196Pro) and partially activating (β3 Cys598Tyr) substitutions is unique in GT. A third unrelated GT patient also possessed the latter mutation, but was compound heterozygote with a separate exon 5 mutation giving a stop codon. HRM analysis permitted the rapid screening of all mutations including the presence of different exon 5 transitions. It also allowed SNP comparison between the two genes in different families. *Conclusions.* HRM provides an accurate and inexpensive same-day tool for studies on mutation inheritance and genetic diagnosis in GT. It detects the penetration of known mutations within families and will speed up prenatal diagnosis of defined GT genotypes.

0708

ANALYSIS OF PERIPHERAL BLOOD CD4$^+$CD25$^+$T CELLS IN PATIENTS WITH ACQUIRED HEMOPHILIA A: IT HAS CORRELATION TO THE CLINICAL SEVERITY AND TREATMENT EFFICACY?

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Background. Immunoregulatory CD4$^+$CD25$^+$ T cells play an important role in the induction and maintenance of peripheral self-tolerance. These professional regulatory cells prevent the activation and proliferation of potentially autoreactive T cells that have escaped thymic deletion. Therefore, we believed that CD4$^+$CD25$^+$ T cells possibly play an important role in acquired hemophilia A (AHA). *Aims.* The aim of this study was to explore the profile and function of CD4$^+$CD25$^+$ regulatory T cells in AHA patients, and to explore the correlation between the level of CD4$^+$CD25$^+$ regulatory T cells and clinical situations. *Methods.* We measured the count of CD4$^+$CD25$^+$ T cells in 10 patients diagnosed as AHA from January 2002 to December 2008 in our hemophilia center. Treg cell numbers were analyzed by flow cytometric analysis in peripheral blood mononuclear cells collected from patients with AHA or healthy donors. According to the titer of inhibitor, we divided these patients into two groups: low responders (≤5 Bethesda units, n=4) and high responders (>5 Bethesda Unit, n=6). Three low responders and all high responders were treated with immunosuppressive therapy such as prednisone (1 mg/kg/d) and/ or cyclophosphamide (200 mg/d), three high responders patients also received high-dose intravenous immunoglobulin (IV IG, 0.4g/kg/d, 5 days). We evaluated the CD4$^+$CD25$^+$ T cells dynamic changes in low responders, high responders and patients obtained partial remission (PR) and complete remission (CR). *Results.* Mean factor VIII level was 5.13 IU/dl (0.16-27.34IU/dl), mean titer of inhibitor was 17 BU (0.85- 65 BU). The percentage of CD4$^+$CD25$^+$ T cells was significantly lower in AHA patients in the active phase or non-remission state (4.23±1.35%). However, the percentage of those cells increased in the patients at the remission phase, including CR and PR (10.96±3.24%), especially after immunosuppressive treatment, which was similar to healthy subjects (13.63±4.56%). CD4$^+$CD25$^+$ T cells counts in high responders (2.75±0.56%) were lower than low responders (6.45±2.05%). But after treatment, all patients'CD4+CD25+ T cells increased, seven patients (all low responders and three high responders) obtained CR with CD4$^+$CD25$^+$ T cells of 12.16±4.11%, three high responders obtained PR with CD4$^+$CD25$^+$ T cells of 8.92±2.96%. *Conclusions.* These results suggested that decreased number of CD4$^+$CD25$^+$ T cells in AHA patients without effective treatment. The number of CD4$^+$CD25$^+$ cells closely correlated with the factor VIII level and inhibitor titer, and would be increased when immunosuppressive treatment was effective. CD4$^+$CD25$^+$ T cells might be one of mechanisms that cause immune regulation dysfunction in AHA, and the count of CD4$^+$CD25$^+$ T cells is considered to possibly be related to the severity of AHA.

0709

SEROPREVALENCE OF HEPATITIS A VIRUS IN EGYPTIAN CHILDREN AND ADOLESCENTS WITH HEMOPHILIA A: SAFETY AND IMMUNOGENICITY OF SUBCUTANEOUS HEPATITIS A VACCINE

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Background. Hepatitis A is a highly contagious infectious liver disease prevalent in Egypt , however hepatitis A vaccine is not routine compulsory vaccine. Transmission of hepatitis A virus (HAV) is primarily via the faeco-oral route; however hemophiliacs are at risk of transmission through clotting factor concentrate, particularly products inactivated by solvent-detergent purification. *Aim.* The aim of this study was to evaluate the seroprevalence of hepatitis A in non vaccinated Egyptian children and adolescents with hemophilia A as well as the safety and immunogenicity of subcutaneous hepatitis A vaccine in hemophilia patients. *Methods.* 182 male children and adolescents (aged 2-18 years) were studied,including 82 patients with moderate and severe hemophilia A (mean age 9.49±4.26 years) and 100 healthy controls (mean age 8.67±3.97 years). All did not receive prior hepatitis A vaccination and had no clinical history suggestive of hepatitis . After informed consent was taken from patients or their guardians, screening for anti-HAV antibody (IgG and IgM) was done by microparticle enzyme immunoassay using Abbott AxSYM analyzer. Seronegative patients received hepatitis A vaccine (HAVRIX,GlaxoSmithKline) at a dose of 720 Elisa Units at 0 and 6 months, given subcutaneous in hemophiliacs (without prophylactic factor replacement) and intramuscular for controls.Seroconversion rate was assessed in vaccinated children 2 months after the second vaccine dose by anti-HAV antibody IgG. Anti-HAV IgM was assessed at month 7 to exclude acquired hepatitis A infection in vaccinated children. *Results.* Seroprevalence of HAV antibody was 87.6% among hemophilia patients and 90% among the control group. Seronegative children(mean age 4.4±3.71 years) were significantly younger than seropositive children (mean age 10.2±3.86 years), P<0.01. Hepatitis A vaccine was given to 10 non immune hemophilia patients (SC) and 10 controls (IM). The vaccine was well tolerated by both groups with minor local side effects including pain in 40% and erythema in 20% of hemophiliacs *vs.* 20% for pain and erythema in the control group. No swelling or bruising was reported. As regards immunogenicity, all patients and controls developed seroconversion 2 months after the second dose ; there was no significant difference between both groups as regards the titre of anti- HAV IgG (351.4±65.0IU/L for hemophiliacs and 367.0±132.3IU/L for controls), P>0.05. *Conclusion.* Hepatitis A virus infection occurs early in life in Egyptian children , necessitating hepatitis A vaccination in early life especially in highly vulnerable patients as hemophiliacs.The vaccine is safe and effective when given subcutaneously in children with hemophilia.

0710

A NOVEL WHOLE PLATELET ELISA FOR THE DIAGNOSIS OF GLANZMANNS THROMBASTHENIA

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Glanzmann's thrombasthenia is a rare autosomal recessive bleeding disorder, caused by a quantitative or qualitative defect of the platelet glycoprotein GPIIb-IIIa receptors with bleeding due to defective platelet haemostatic plug formation. There are various methods which are used for diagnosis of Glanzmann's thrombasthenia. Some of the methods are clot retraction, flow cytometry analysis, platelet aggregation studies, mutation analysis, dot blot analysis. These methods have their own advantages and disadvantages and these methods have their own flaws in detection of heterozygotes in Glanzmann's thrombasthenia. In this study, we describe an experiment of standardization of enzyme linked immunosorbent assay (ELISA) for detection of index cases as also heterozygotes in Glanzmann's thrombasthenia and then diagnosis of known and unknown heterozygotes in Glanzmann's thrombasthenia. The principle of sandwich ELISA was used as the final protocol during standardization of the above technique. The receptor glycoprotein GPIb/IIIa present on the surface of platelets acted as 'target antigens'.Fresh blood samples were obtained from the patients as well as from the controls and platelets isolated by using PGE1 and coated on to the wells. On the first day, 100 μL of 1:100 Anti CD36 dilutions was added to each micro titre well and then this plate was properly covered with a lid and incubated overnight at 40 C. On the second day the platelets are isolated and then resuspended in the RCD buffer and the count adjusted to 70,000/μL using the coulter counter. Subsequently the dilutions of the neat concentration of the platelets are carried out, the incubated plate is then flicked to remove excess of anti-CD36, and 100μl of each dilution of the platelet is added to the wells and incubated overnight at 40 C. On the third day, the wells were washed once using PBS-Tween20 and 100 μL of 1:100 echistatin conjugated with alkaline phosphatase was added to each well and then incubated for an hour at room temperature. After an hour the wells were washed once and 100μl of substrate is added and incubated at room temperature in dark for an hour. The OD was checked at 405 nm using an ELISA plate reader.The present study is the first ever to diagnose Glanzmann's thrombasthenia and the heterozygotes in GT using a whole platelet ELISA technique. From this standardized protocol, we were able to diagnose 8 known heterozygotes 2 unknown heterozygotes for GT. 30 controls were used for this study. The diagnosis of a heterozygote carrier of GT or variant type II GT can be made with a high probability using this ELISA.

0711

HIGH PREVALENCE OF ARGININE HOT SPOT MUTATIONS IN INDIAN SEVERE VWD PATIENTS

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Von Willebrand disease (VWD) is the most frequent inherited bleeding disorder caused by qualitative and or quantitative abnormalities of the von Willebrand factor (VWF). Mutation detection in VWF gene is quite challenging due to its large size (180 Kb), heterogeneous nature of mutations and the presence of a partial unprocessed pseudogene in chromosome 22 with 97% homology. In this study we adopted an initial strategy for screening for 11 CGA Arginine codons of the VWF gene in 78 unrelated severe VWD patients from all over India. 15 nonsense mutations were detected in exon 8, 9, 18, 28, 31, 43 and 45 by PCR-RFLP technique. Direct mutation detection by RFLP analysis in hot spot regions is the most simple, less time consuming and cost effective method especially in VWF gene which comprises of 52 exons and screening for mutation in type 3 VWD is difficult since mutations are not confined to the specific regions as in case of other VWD subtypes. We could detect 11 homozygous (1 each in exon 9,18, 28 and 45, 2 in exon 8, 43 and 3 homozygous in exon 31) and 4 (1 in exon 10, 18 31 and 45) heterozygous mutations by this relatively simple technique and thus report high prevalence (21%) of arginine hot spot mutations in Indian population. The study is significant in that most of the diagnoses are being based on the indirect method of gene tracking analysis which has limitations like noninformativeness of the markers, need for the index case and so on. Direct mutation detection by simple PCR RFLP technique can give an accurate diagnosis in approximately around 1/5 of the severe VWD cases without going for the more extensive and expensive mutation screening and sequencing techniques.

0712

LIVER TRANSIENT ELASTOGRAPHY (FIBROSCANNING) IS USEFUL IN THE ASSESSMENT OF LIVER FIBROSIS IN HEPATITIS C INFECTED PATIENTS WITH INHERITED BLEEDING DISORDERS

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The accurate assessment of the degree of hepatic fibrosis is crucial for treatment decisions and prognosis in patients with inherited bleeding disorders infected with Hepatitis C (HCV). Transient elastography (fibroscanning) has recently been developed as a non-invasive alternative to liver biopsy. The technique assesses liver stiffness, a reflection of the degree of hepatic fibrosis. The introduction of fibroscanning has encouraged patients who have previously had concerns over the safety of liver biopsy to request active HCV management. 34 hepatitis C-infected haemophilia patients (33M, 1F) with a median age of 43 years (range 26-70) were assessed by fibroscan. Diagnoses: Haemophilia A(29), Haemophilia B(2), von Willebrand's disease (2, 1 type 2A, 1 type 3).12 patients were coinfected with HIV. 23 patients had not previously undergone HCV treatment. 10 of 11 patients who had previously received treatment with ribavirin and PEG interferon had persistent HCV infection. The breakdown of scores in the patients was as follows: score <7.0 (no or very mild fibrosis - Metavir classification F0-F1) n = 13, score 7.0 to 9.5 (significant fibrosis F2) n = 10, score >9.5 to 12.5 (severe fibrosis F3) n = 3, score >12.5 (cirrhosis F4) n=4. A fibroscan score was unobtainable in four patients because of a high BMI. Therefore encouragingly 43% of this cohort had fibroscan scores consistent with no or very mild fibrosis at least 30 years from acquisition of HCV

infection. Fibroscanning is a non-invasive alternative to liver biopsy in the assessment of HCV associated fibrosis in patients with hereditary bleeding disorders. The fibroscan score provides valuable information in the management of this patient group enabling decisions to be made such as whether or not to embark on eradication therapy or to await the new anti HCV medications.

0713

PROTHROMBIN MUMBAI: NOVEL CYS90PRO MUTATION IN A FAMILY

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Coagulation factor II (F2) is proteolytically cleaved to form thrombin in the first step of the coagulation cascade which ultimately results in the stemming of blood loss. F2 also plays a role in maintaining vascular integrity during development and postnatal life. Mutations in F2 leads to various forms of thrombosis and dysprothrombinemia. Thrombin, which cleaves bonds after Arg and Lys, converts fibrinogen to fibrin and activates factors V, VII, VIII, XIII, and, in complex with thrombomodulin, protein C. We report here a novel mutation in Human Prothrombin (Factor II) gene in a family originating in Nepal. A four and half months old female child, born of non-consanguinous marriage to a 25 years old lady, was referred as a case of bleeding disorder with history suggestive of acute intracranial haemorrhage. At 21 hrs of life she developed hematemesis and was treated with Vit K for 3 days. Baby developed bleeding from umbilical stump for which surgical ligation was done and FFP and Vit K was given by the treating clinicians. FXIII clot stability test was normal. At 3 months of life, baby developed Intracranial bleed. CT brain showed Rt SDH with marked mass effect and Rt. occipital lobe hematoma. Prothrombin Time (PT) was 27/14.5, APTT was 40/23-35, Thrombin Time (TT) was 18/15 and Fibrinogen was 222gm/dl (normal). Factor II (Prothrombin) was found to be <1% on assay, with other factors within normal limits. DNA sequencing of all 14 exons of Factor II gene showed a G>C homozygous variation in exon 4, which caused a Cys90Ser mutation. Both the parents showed a heterozygous G>GC change. Bioinformatics tools such as the Panther classification system (Protein ANalysis THrough Evolutionary Relationships), Polyphen (Polymorphism phenotyping) and SIFT (Sorting Intolerant from Tolerant) suggested that this is a deleterious variation and has not been reported in Ensembl genome browser to detect reported nucleotide variations. Clustal W multiple sequence alignment analysis was done to look for the conservation status of the residues across mice to primates and humans. It showed that Cys90 is highly conserved across these species. Uniprot consortium shows that residue Cys90 lies just outside the Gla (gamma-carboxy-glutamate) domain and is involved in a disulfide bond with Cys103. The severe clinical manifestations that the baby had correlate well with the mutation detected and the deleterious effects it is predicted to cause. This family has been subsequently counseled for a possible antenatal diagnosis for the next pregnancy.

0714

DIRECT DETECTION OF HEMOPHILIA B F9 GENE MUTATION WITH MULTIPLEX PCR AND CONFORMATION SENSITIVE GEL ELECTROPHORESIS

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Background. The F9 gene is known to be the causative gene for hemophilia B, but unfortunately the detection rate for restriction fragment length polymorphism (RFLP) -based linkage analysis is only 55.6%. Direct DNA sequencing can detect 98% of mutations, but this alternative procedure is very costly. *Aims.* Here we conducted multiplex PCR-conformation sensitive gel electrophoresis (CSGE) screened DNA sequencing for the F9 gene and compared the results with direct sequencing in terms of accuracy, cost, simplicity and time consumption. *Methods.* A total of 27 unrelated hemophilia B patients was enrolled. Direct DNA sequencing was performed for 27 patients by a separate institute, and multiplex PCR-CSGE screened sequencing in our laboratory. Results of the direct DNA sequencing were used as a reference and the results of the multiplex PCR-CSGE screened sequencing were compared with them. For the patients whose mutation was not detected by the 2 methods, multiplex ligation-dependent probe amplification (MLPA) was conducted. *Results.* With direct sequencing, the mutations could be identified from 26 patients (96.3%), whereas for multiplex PCR-CSGE screened sequencing, the mutations could be detected of 23 patients (85.2%). One patient's mutation was identified by MLPA. A total of 21 different mutations was found among the 27 patients. Multiplex PCR-CSGE could reduce the cost of detection by 55.7% compared with direct DNA sequencing, but this technique was more labor-intensive and time-consuming as it required an extra day. Summary. Multiplex PCR-CSGE screened DNA sequencing detected 88.9% of mutations and reduced costs by 55.7% compared with direct DNA sequencing. But it was more labor-intensive and time-consuming.

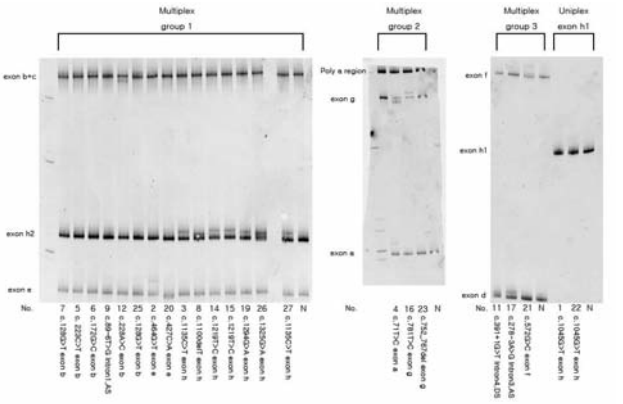

Figure 1.

0715

GENOTYPIC AND PHENOTYPIC CHARACTERIZATION OF FACTOR VII DEFICIENCY PATIENTS FROM NORTH-EAST HUNGARY

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Background. Congenital Factor VII (FVII) deficiency is one of the most frequent disorders among rare coagulopathies. The molecular genetic background is heterogenous; no so-called mutation hot spot can be identified within the gene of FVII (F7). No report has been available so far on the mutation profile of Hungarian patients. *Aims.* The aim of the study was to give an overview on the spectrum of mutations of F7 and on genotype-phenotype associations in Hungary. *Methods.* We analyzed 14 unrelated patients with FVII deficiency and their family members living in North-East Hungary for mutations in F7 by direct DNA sequencing of the coding region of the gene including exon-intron boundaries. Clinical symptoms, hemostasis laboratory results and treatment modalities were recorded and related to molecular genetic findings. *Results.* A total of 7 different mutations (6 point mutations and 1 small deletion) were identified, of which 2 were novel (p.E7G and p.C368F). The p.E7G mutation affects one of the glutamic acid residues involved in the gamma-carboxylation process suggesting functional defect of FVII. The p.C368F mutation locates in the catalytic domain where Cys368 forms a disulfide bridge with Cys340. The amino acid exchange to Phe368 suggests the disruption of this disulfide bridge and misfolding of the protein. Patients with severe laboratory phenotype (FVII activity < 1%) were homozygotes or compound heterozygotes. Patients or family members with heterozygous mutations exhibited mild symptoms or were silent carriers. Combined coagulopathy was found in two families, where besides FVII deficiency heterozygous factor X deficiency was also diagnosed. *Conclusion.* The genetic background of FVII deficiency is heterogenous even in the presented small cohort of patients from North-East Hungary. Most of the patients carried more than one causative mutation. In some patients a weak correlation could be demonstrated between the severity of bleeding symptoms, laboratory phenotype and the underlying genetic alterations.

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0716

IDENTIFICATION OF FUNCTIONAL DOMAINS AND NOVEL BINDING PARTNERS OF STIM PROTEINS

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Background. Store-operated $Ca2^+$ entry, which is triggerd by the depletion of endplasmic reticulum (ER) $Ca2^+$ stores, is essential for the refill of $Ca2^+$ in ER and the cytokine/hormone-induced sustained increase of intracellular $Ca2^+$. In immune cells, stromal interaction molecule (STIM) proteins, which are unique sensors of ER $Ca2^+$ changes, have been believed to initiate store-operated $Ca2^+$ entry and to play pivotal roles in various cellular events, such as gene expression, cell activation, and differentiation. Indeed, the destruction of both STIM1 and STIM2 genes leads to the impairment of regulatory T-cell development. In addition, patients with homozygous nonsense mutations in STIM genes show phenotypes of immunodeficiency and/or autoimmunity. Thus, there is accumulating evidence that STIM proteins are involved in immune systems. Although expanding information is available for biochemical and physiological properties of STIM proteins, little is known about the domain functions of STIMs and their association molecules, which could help us understand not only why STIMs can display their specific functions but also what molecules control their localization and protein content in cells. AIMS Aims of this study are to clarify domain functions of STIM1 and to identify STIM1- associated molecules. Methods/Results. We constructed a series of deletion mutants of STIM1; STIM1(EX, EX+TM, EX+TM+CC, Full), where "EX" means extracellular domain, "TM"means transmembrane domain, "CC"means coiled-coil domains, and "Full"means full length of STIM1. First we examined protein trafficking of each mutant. After surface proteins on each transfectant were labeled with biotin, the cell lysates were analyzed with western blot using HRP-streptavidin. STIM1(EX+TM) was massively expressed on the cell surface, while surface-expressed STIM1(EX+TM+CC) and STIM1(Full) were only faintly detected. In addition, analysis with confocal microscopy revealed that only STIM1 mutants containing the coiled-coil domains, STIM1(EX+TM+CC) and STIM1(Full), aggressively accumulated in ER. Then we evaluated protein stability of each STIM1 mutant with 35S-methionine pulse-chase experiments. Estimated half life of STIM1(Full) was approximately 19 hours, similar to that of STIM1(EX+TM+CC). However, STIM1(EX+TM) disappeared more rapidly. Therefore, the coiled-coil domains of STIM1 is likely to yield its ER-retention and protein stability. To identify STIM1 associated molecules comprehensively, we performed co-immunoprecipitation experiments combined with mass spectrometry. After 293T cells were transfected with STIM1(Full)-FLAG, the cell lysates from each transfectant were subjected to immunoprecipitation with anti-FLAG antibody. After visualization of the immunoprecipitated proteins in the gel with silver staining, several specific bands were detected. Each specific band was subjected to mass spectrometry analysis. This screening identified calnexin, an ER-resident chaperone as a novel STIM-associated protein. The association was also confirmed with co-immunoprecipitation experiments. Importantly, the interaction between STIM1 and calnexin was independent on N-glycosylation, which calnexin generally recognizes. Therefore, STIM1 is likely to bind to calnexin in a specific manner. Summary/Conclusions. Our data suggest that the coiled-coil domains of STIM1 is involved in its ER-retention and protein stability and that calnexin is a novel STIM1-associated molecule. These findings might be useful to discuss unique characteristics of STIM1 proteins.

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A CELL-DEATH-DEFYING FACTOR, ANAMORSIN, CONTRIBUTES CELL GROWTH THROUGH INACTIVATION OF P38 MAPK

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Background. Anamorsin (also called CIAPIN-1) is a cell-death-defying factor, which was originally isolated as a molecule that conferred resistance to apoptosis induced by growth factor starvation. Anamorsin is ubiquitously expressed in various organs, including hematopoietic tissues like bone marrow, spleen, and thymus. Anamorsin-deficient mice die in late gestation. Anamorsin-deficient embryos are anemic and the size of the embryos is very small. It is thought that anamorsin plays a crucial role in hematopoiesis during late and/or terminal stages of differentiation. Anamorsin does not show any homology to known apoptosis and cell growth regulatory molecules such as Bcl-2 family, caspase family, or signal transduction molecules.(J Exp Med 199: 581-592, 2004) However, the precise biological effects of anamorsin remained unclear. *Methods and Results*. In an attempt to clarify the biological effects of anamorsin, we here tried to determine the signaling pathways by using murine embryonic fibroblast (MEF) cells produced from E-14.5 anamorsin-deficient or wild type embryos. The proliferation of anamorsin-deficient MEF cells was quite reduced compared with the wild type MEF cells. It is found that the phosphorylation status of ERK1/2, NFκB, and AKT were similar both in anamorsin-deficient MEF cells and wild type MEF cells, while p38 MAPK was more phosphorylated in anamorsin-deficient MEF cells than wild type MEF cells. P38 MAPK was also more phosphorylated in the stimulation of PMA, the PKC activator. Furthermore, the expression of cyclin D1, the target molecule of p38 MAPK, was down-regulated in the anamorsin-deficient MEF cells. The p38 MAPK inhibitor, SB203580, up-regulated the expression of cyclin D1 and partially restored the proliferation of the anamorsin-deficient MEF cells. Based on these data, it was thought that p38 MAPK activation reduced the cyclin D1 expression, thereby, resulting in the growth retardation of the anamorsin-deficient MEF cells. Conclusion. P38 MAPK, the stress activated MAPK, has been known to link to cell differentiation, growth inhibition and apoptosis, and also to be essential for survival in response to various stimuli. Moreover, p38 MAPK can contribute to the induction of G1/S and G2/M cell cycle check point. The growth of the anamorsin-deficient mice is quite retarded, and the hematopoiesis of the mice is also impaired. Our study suggests that p38 MAPK might play important roles in the cell proliferation, survival, and differentiation of the anamorsin-deficient cells.

0718

MESENCHYMAL STROMAL CELLS INDUCE A CD4+CD25HI REGULATORY T-CELLS WITH HIGHER LEVELS OF CD73/ECTO-5-NUCLEOTIDASE THAN NATURALLY EMERGING CD4 CD4+CD25HI REGULATORY T-CELLS

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Background. Mesenchymal stromal cells (MSCs) exert an immune regulatory function and suppress T-cell proliferation *in vitro* and *in vivo*, but at present, the mechanisms underlying this property are not completely known. One of the ways by which MSCs may modulate immune responses is by the induction of regulatory T cells (Treg). Generation of adenosine by CD73 mediates the imunosupressive capacity of naturally emerging Treg. Adenosine exerts several physiological effects though the interaction with four adenosine receptors. Moreover, adenosine levels are controlled by its convertion to inosine by the enzyme Adenosine Deaminase (ADA). Furthermore, biding of ADA to CD26 is capable of reducing the local concentration of adenosine and protects T cells from suppression. Several studies clearly show the role of adenosine as a T-cell immune modulator, mainly by activation of A2A receptor (AR A2A). Besides, AR A2A signalizing induces IL-10 production, an important immunoregulatory cytokine. *Aims.* To evaluate the involvement of adenosine signaling components in the immunomodulation promoted by MSCs. *Methods.* Peripheral blood $CD3^+$ T cells from 3 individuals were activated by anti-CD3/CD28 beads, cultured for 5 days either in the presence or in the absence of MSC (5:1). Following this period, $CD4^+$ cells were immunomagnetically purified and evaluated by RT-PCR for the expression of ADA, AR A2A and IL-10. In addition, periph-

eral blood CD3+ T cells from other 3 individuals were similarly activated and cultured. Following a 5 day period, ADA activity and adenosine levels were analyzed in culture medium by colorimetric assay and high performance liquid chromatography (HPLC), respectively. Percentage of CD26+ and CD73+ cells was accessed by flow cytometry on T cells cultivated or not with MSC. *Results.* As expected, proliferation of T cells co-cultured with MSC was significantly inhibited (BrdU incorporation assay). In line, T cells co-cultured with MSC showed higher level of AR A2A and IL-10, and lower level of ADA than lymphocytes cultured alone. Moreover, ADA activity was higher in culture medium from T-cells cultivated with MSCs and despite that, the extracellular levels of adenosine were higher in T-cells cultivated with MSCs compared to T cells cultivated alone (although not statistically significant). The percentage of CD26 in CD4 T-cells co-cultured with MSC was lower than in T-cells cultivated alone, indicating low local ADA concentration, thus favoring adenosine suppressive function. Interestingly, Tregs (induced by MSCs or naturally emerging after activation in the absence of MSC) displayed high levels of CD26, probably as a protective mechanism to locally counteract their suppression by adenosine. Finally, the percentage of CD4+ T-cells expressing CD73 increased significantly upon co-culture, indicating a shift toward adenosine production. Moreover, the percentage of CD73+ cells was higher among Treg induced by MSC than among naturally emerging Treg. *Conclusions.* We demonstrate for the first time that MSCs downmodulates the expression of CD26 in effector CD4+ T-cells, while inducing the generation of Treg with higher proportions of cells co-expressing CD73 or CD26, indicating an important role of adenosine signaling in the immunosupression mediated by MSC.

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CALPAIN IS INVOLVED IN PTP1B MODULATION BY ERYTHROPOIETIN IN TF-1 AND DIFFERENTIATED UT-7 CELLS

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Background. The central role played by tyrosine phosphorylation of erythropoietin receptor (EpoR) in cell activation by erythropoietin (Epo) has focused attention on protein tyrosine phosphatases (PTPs) as candidates implicated in the pathogenesis of the resistance to therapy with human recombinant Epo. Prototypic member of the PTP family is PTP1B, a widely expressed non-receptor PTP located both in cytosol and endoplasmic reticulum membrane, which has been implicated in the regulation of signaling pathways involving tyrosine phosphorylation induced by growth factors, cytokines and hormones. In previous reports we have shown that PTP1B expression, activity and phosphorylation are reciprocally modulated by Epo in undifferentiated UT-7 cell line. However, no information is available with respect to the modulation of this phosphatase in non-Epo depending cells or at late stages of erythroid differentiation. *Aim.* To characterize the relationship between Epo and PTP1B expression and to investigate a probable link between changes in PTP1B and intracellular calcium levels modulated by Epo. *Methods.* The TF-1 human cell line was used as a model of cells that can be grown with GM-CSF or Epo while the Epo-dependent UT-7 cells were induced to erythroid differentiation with hemin. Controls: TF-1 cells cultured in the presence of GM ad undifferentiated UT-7 cells cultured with Epo. Epo was added to Epo/serum-deprived cells for 18 h and after different periods, cells were lysed and total proteins and RNA were obtained to be analyzed by Western blotting and Real Time PCR, respectively. Immunoprecipitates with anti-PTP1B were subjected to a PTP1B activity assay using p-nitrophenylphosphate as substrate. Experiments with ionophore A23187 and calpeptin were performed to determine calpain involvement in PTP1B modulation by Epo. Flow cytometry was used to quantify calcium response to Epo. *Results.* A significantly increased level of PTP1B mRNA was observed in TF-1 and differentiated UT-7 cells either permanently maintained with Epo or Epo-stimulated for 24 h ($P<0.05$, n=4). This increment correlated with the highest level of PTP1B activity under the same conditions. Besides, an additional 46 kDa PTP1B isoform was observed in TF-1 cells and in hemoglobinized UT-7 cells cultured in the presence of Epo. After subcellular fractionation, this protein was found to be attached to the membrane fraction as the 50 kDa PTP1B isoform. Cell pre-treatment with calcium ionophore showed that a calcium-dependent protease was involved in the PTP1B cleavage. The addition of calpeptin to the experiments prevented PTP1B degradation, demonstrating that calpain was responsible for this Epo modulation. Flow cytometry analyses showed an Epo-induced intracellular calcium increase which was maintained after 60 min-Epo stimulation in TF-1 and differentiated UT-7 cells but not in UT-7 control undifferentiated cells. *Conclusions.* Results demonstrated an Epo-induced cleavage of PTP1B, resulting in a 46 kDa isoform attached to cell membrane which is associated with increased PTP1B enzymatic activity. Calpain was found to be responsible for this differential cleavage being the protein activation presumably a consequence of the intracellular calcium concentration change induced by Epo. These findings suggest a novel pathway of Epo - induced PTP1B regulation involving calcium, an already well known intracellular mediator.

0720

RERVERSE SIGNALING B CELL-ASSOCIATED B7-H1 INDUCES CLASS SWITCHING TO IGG1 AND IGG3

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Background. B7-H1 (CD274), a B7 family ligand, delivers an inhibitory signal to its counter-receptor PD-1 and as-yet-unidentified receptor(s), leading to negative T cell immunity. Recent studies suggest that B7-H1 also receives a signal from its counter-receptor, rendering B7-H1+ cancer cells resistant to cytotoxic T cell killing activity and inducing proliferation and subsequent apoptosis of B7-H1+ T cells. Aim. We investigated the role of reverse signaling through B cell-associated B7-H1 (B-B7-H1) in proliferation, apoptosis, and immunoglobulin class switching of B cells. *Methods*. B7-H1+ tonsillar B cells were separated from B7-H1- B cells, and then were grown in 96 well plate coated with control Ig or anti-B7-H1 in the presence of anti-CD40 and IL-4. Total RNA was isolated from each B cell population and then subjected to RT-PCR for AID gene and , germline IH-CH transcripts of and mature VHDJH-CH transcripts. In some experiments, culture supernatant was harvested and subjected to ELISA for Ig isotypes. For examination of apoptosis of B cells cross-linked with anti-B7-H1, tonsillar B cells were incubated in 96 well plate plate coated with control Ig or anti-B7-H1 in the presence of anti-CD40 and IL-4, and then subjected to Annexin-V staining. *Results.* With plate-coated anti-B7-H1, B7-H1+ B cells exhibited upregulation of activation-induced cytidine deaminase (AID) gene transcription and class switch recombination from Cm to Cg1 and Cg3. B-B7-H1-mediated IgG productivity was positively correlated with the percentage of B7-H1+ B cells among the CD19+ B cells. B-B7-H1 cross-linking also induced apoptosis-promoting genes such as FasL, Bad, and Bid, but not anti-apoptotic Bcl-2, and lead to increased apoptosis in B7-H1+ B cells compared with B7-H1- B cells. In contrast to T cell-associated B7-H1, the engagement of B-B7-H1 was not responsible for B cell proliferation. *Conclusions.* These results indicate that reverse signaling through B-B7-H1 induced class switching to IgG1 and IgG3 and apoptosis, but was not involved in B cell proliferation.

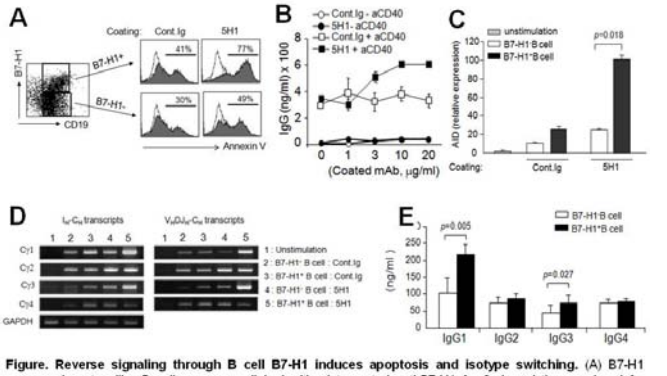

Figure. Reverse signaling through B cell B7-H1 induces apoptosis and isotype switching. (A) B7-H1 expressed on tonsillar B cells was cross-linked with plate-coated anti-B7-H1 for 3 d, and then analyzed for apoptosis with Annexin V staining. (B) Tonsillar B cells were treated as in (B) for 10 d in the presence of anti-CD40 and IL-4, and then ELISA was performed for IgG production from B cells. (C, D) B7-H1+ B cells were separated from B7-H1- B cells by MACS sorting procedure, and then treated as in (B) for 3 d. Total RNA was purified for RT-PCR for AID gene, germline I_H-C_H transcripts of and mature V_HDJ_H-C_H transcripts. (F) ELISA analysis of Ig isotypes in various B cell preparations engaged with control Ig- or 5H1-coated microbeads for 10 d.

Figure.

0721

IMATINIB-INDUCED APOPTOSIS; A POSSIBLE LINK TO TOPOISOMERASE ENZYME INHIBITION

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Background. Chronic myeloid leukemia (CML) is a hematological malignancy resulting from a chromosomal translocation between long arms of 9. and 22. chromosomes that generates BCR/ABL fusion protein. Imatinib, a specific BCR/ABL inhibitor, is commonly used for the treatment of CML. Recent studies showed that imatinib has cytotoxic and apoptotic effects on BCR/ABL negative cancers. We have previously reported that imatinib induces apoptosis by increasing intracellular concentrations of apoptotic ceramides via generation and accumulation of ceramides through upregulating ceramide synthase genes and downregulating anti-apoptotic sphingosine kinase-1 gene. We have recent evidences indicating that imatinib induces autophagy, programmed cell death-II, by increasing the expression levels of Atg5, Atg6, and LC-3 genes in a dose-dependent manner (unpublished data). However, the mechanisms of imatinib induced apoptosis in BCR/ABL negative cancers is yet to be clarified. Numerous natural products and synthetic compounds having anti-cancer potential were shown to exert their functions via interfering with normal DNA topoisomerase functions. *Aims.* In this study, we examined the effects of imatinib on tumor cell killing and topoisomerase I and topoisomerase II enzyme activities. *Methods.* The human K562 cells were grown in RPMI1640 medium containing 10% FBS and 1% penicilline-streptomycine. Cytotoxicity analyses of imatinib were conducted by XTT cell proliferation assay. Changes in caspase-3 enzyme activity were determined using the caspase-3 colorimetric assay. The mitochondrial membrane potential (MMP) was measured using JC-1 MMP detection kit. Topoisomerase I plasmid supercoil relaxation assays and topoisomerase II decatenation assays were conducted in response to imatinib. *Results.* The results revealed that imatinib induces apoptosis and inhibits cell proliferation in a dose-dependent manner. We biochemically addressed plasmid DNA nicking and DNA catenation that rely on the ability of topoisomerase I to relax sc DNA and topoisomerase II to decatenate minicircle DNA substrates, respectively. The interference by imatinib was monitored as faster-migrating substrate band in plasmid relaxation method. Importantly, the results demosntrated that imatinib inhibited topoisomerase I and topoisomerase II enzymes activities in a dose-dependent manner *in vitro*, suggesting a possible link in the mechanism of imatinib-induced tumor cell-killing. *Conclusion.* Taken together, this is the first study proposing a potential link between apoptotic induction and topoisomerase interference by imatinib. The results of this study may help in understanding of how imatinib induces apoptosis and inhibits cellular proliferation in both BCR/ABL negative and positive cancers and strengthens its application potential.

0722

COMPARISON OF NEUROPROTECTIVE AND ERYTHROPOIETIC EFFECTS OF ERYTHROPOIETIN AND CARBAMYLATED-ERYTHROPOIETIN IN DIFFERENT CELL SYSTEMS

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Background. In addition to its well-known hematopoietic effects, erythropoietin (Epo) also has neuroprotective properties. However, hematopoietic side effects are unwanted for neuroprotection, underlining the need for Epo-like compounds with more selective neuroprotective action. One such compound extensively assayed is the chemically modified Epo-derivative carbamylated erythropoietin (cEpo), which in experimental assays cEpo has demonstrated non-hematopoietic tissue protection without significant erythropoietic effect. However, less is known about cellular mechanisms of action. *Aims.* To compare the neuroprotective action between Epo and cEpo and to distinguish their effects upon erythropoietic cells. *Methods.* Cell viability/proliferation was analyzed by the MTT assay and apoptosis by fluorescent microscopy after Hoechst staining. Models: Cells of neuronal origin: SH-SY5Y cells were cultured in E-MEM:Ham-FBS. Cells of ability to erythroid differentiation: a) Epo-dependent UT-7 cells were cultured in IMDM-FBS with Epo, b) Epo-independent K562 cells were grown in RPMI-1640-FBS and differentiated to erythroid lineage with hemin, and c) physiological colony forming units-erythroid (CFU-E) from Balb/c mice were evaluated by bone marrow cell cultures in methylcellulose-medium. After 48 h, 2,7-diaminofluorene reaction was performed and hemoglobinized colonies were counted. *Results.* Epo and cEpo acted in similar way (no significant differences) to prevent apoptosis induced by either staurosporine (STP) or TNF-α in SH-SY5Y cells (Control 8±1.5%; STP 44±5.2%; Epo-STP 12±2.2%; cEpo-STP 12±1.7%; TNF 32±1.7%; Epo-TNF 9±0.9%; cEpo-TNF 10±2.9%; n=4). Moreover, assays in the presence of inhibitors showed that cell activation by Epo and cEpo involves similar signaling pathways mediated by PI3K and Jak2. Instead, Epo but not cEpo overcame apoptosis induced by TNF-alpha in erythroid differentiated K562 cells (Control 8±2.3%; TNF 17±2.7%; Epo-TNF 8±1.0%; cEpo-TNF 18±1.5%, Epo-TNF *vs.* Epo $P<0.05$, n=4). Different from Epo, cEpo did not prevent apoptosis in cultures of UT-7 cells (Without Epo 39±5.8%; Epo 4±0.3%; cEpo 34±6.2%, n=4). Besides, Epo but not cEpo acted as growth factor in the development of mice CFU-E (Figure). To investigate probable competition between Epo and its carbamylated derivative, cEpo dose-response analysis were performed in the presence of Epo. Results showed that CFU-E growth in the presence of equal amounts of Epo and cEpo (20 ng) did not change the effect of Epo alone. Interestingly, higher amounts of cEpo (200 ng) inhibited the effect of Epo (20 ng) when both compounds were added simultaneously to cultures, but this inhibitory effect disappeared when cEpo was added 60 min after cellular stimulation by Epo (Figure: * Significant differences *vs.* [Without Epo] or [cEpo] or [Epo+cEpox10], $P<0.001$, n=10). *Conclusions.* This work adds new information about the neuroprotective and non-erythropoietic functions of cEpo. Similar mechanisms of action as anti-apoptotic factors in neuronal cells may be suggested for both compounds. However, results showing that the interference of high level of cEpo only occurred at early stages during the interaction of Epo with its erythroid target cell arose for the first time the possibility that cEpo block or modulate the activation of the Epo receptor. This finding may become crucial because it has been reported that relatively high doses of cEpo are needed to act as a neuroprotective factor.

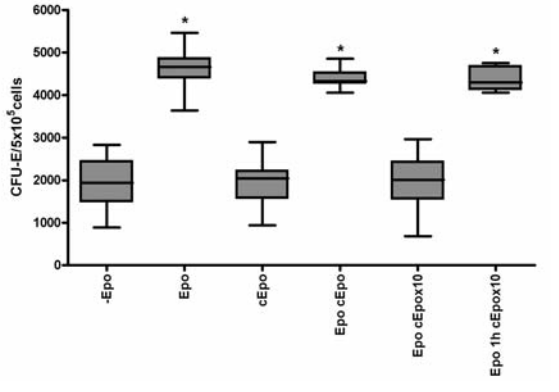

Figure 1. Effect of Epo and cEpo on CFU-E development.

0723

KIR GENOTYPING OF ITALIAN PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA PATIENTS

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Background. Paroxysmal Nocturnal Haemoglobinuria (PNH) is a very rare haematopoieis disorder characterised by the expansion of a stem cell bearing a somatic mutation in the phosphatidyl-inositol glycan-A (PIG-A) gene, which is involved in the biosynthesis of the glycosyl-phosphatidyl-inositol (GPI) anchor. Haemolytic anaemia, thrombophilia and cytopenia characterise this disease. A number of data suggest the inability of PIG-A mutation to account alone for the clonal dominance of the

GPI-defective clone and for the development of PNH. In this context, the occurrence of immune-mediated mechanisms have been hypothesized. *Aims.* Our previous data (Hum Immunol 2008, 69:202-6) revealed the association of PNH with the HLA class I haplotype B*1402, Cw*0802 as well as with the extended Mediterranean haplotype A*33, B*1402, Cw*0802, DRB1*0102. NK cells are critical components of the innate immune response. They may also drive, shape and regulate the activity of the adaptive immune compartment. Killer-cell Immunoglobulin like Receptors (KIR) represent, with their known HLA ligands, a key component in the regulation of NK response. To analyse the biological mechanisms underlying PNH pathogenesis we are addressing the analysis of KIR genotype, whose role in the regulation of immune response and self-tolerance has been established. *Methods.* KIR distribution in 53 patients affected by PNH (estimated to represent almost the half of all Italian PNH patients) and in 64 controls of the same ethnical origin was analysed by PCR-SSP typing. In order to increase the number of controls bearing the HLA class I haplotype (B*1402,Cw*0802), 14 normal donors bearing such HLA haplotype have been selected and additionally enrolled in the study. Association has been tested by two-tailed Fisher's exact test with software InStat 3.0 (GraphPad Software Inc., San Diego, California, USA). Results. No significant associations with the KIR haplotype A or B as well as with specific KIR alleles has been observed. Independent segregation of HLA (6p21) and KIR (19q13.4) genes raises the possibility that any given individual may express KIR molecules from which no ligand is present or vice versa. The analysis of KIR-HLA matching in PNH patients, as compared with controls, showed significant differences only in the 3DL1/Bw4 matching. Indeed, a significant decrease in 3DL1/Bw4 mis-matching has been appreciated in the B*1402,Cw0802 subgroup (3DL1 without Bw4 was observed in 50% of controls as compared with 8.33% of PNH patients; P<0.05). When considering the occurrence of weak interactions, as represented by the presence of T80 Bw4 alleles, such observation become more consistent (72.22% in controls *vs.* 19.66% in PNH patients; P<0.01). In addition, a significant decrease in genotypes showing 3-4 activating genes (46.67% *vs.* 21.95%; P<0.01) accompanied by an increase in the frequency of genotypes showing 1-2 activating genes (35% *vs.* 56.09%) has been observed in B*1402,Cw0802- PNH patients, as compared with controls. Conclusions. This study suggests that a genotype showing higher NK activation threshold (less activating genes together with more effective 3DL1/Bw4 inhibitory pathway) characterises our cohort of PNH patients. This association may provide new insights into the autoimmune pathogenesis of PNH.

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EXPRESSION ANALYSIS OF 84 GENES INVOLVED IN DIFFERENT SIGNALLING PATHWAYS OF CANCER IN CHRONIC MYELOID LEUKEMIA CELLS IN RESPONSE TO NILOTINIB

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Background. Chronic myeloid leukemia (CML) is a hematological malignancy arising from a reciprocal translocation between long arms of chromosomes 9 and 22. The resulting BCR/ABL fusion protein is a strong oncogenic protein that regulates cell growth and proliferation, apoptosis and senescence, migration and adhesion. Imatinib was the first tyrosine kinase inhibitor for the treatment of CML. Although there were significant hematologic and cytogenetic responses to imatinib, resistance cases were observed in patients during treatments and this was the major drawback of imatinib treatment. After identification of the mechanisms of imatinib resistance, a more effective anticancer agent, nilotinib, was developed and started to be used for the treatment of Philadelphia chromosome positive hematological malignancies. *Aims.* In this study, we aimed to examine the molecular mechanisms of nilotinib-induced cell death in addition to inhibition of BCR/ABL in K562 chronic myeloid leukemia cells. *Methods.* Antiproliferative effects of nilotinib were determined by XTT cell proliferation assay. Increasing concentration of Nilotinib (20 and 50 nM) were applied to K562 cells. After 72 hours incubation, total RNAs were isolated and converted to cDNA. Changes in expression levels of 84 genes involved in apoptosis, cell cycle, senescence, adhesion, invasion, metastasis, angiogenesis, transcription factors, and signal transduction molecules were examined by PCR array. *Results.* There were 40 and 55% decreases in proliferation of K562 cells in response to 20 and 50 nM nilotinib, respectively, as compared to untreated controls. Gene expression results revealed that 50 nM nilotinib application resulted in more than 4-fold increases/decreases in expression levels of 41/6 genes as compared to untreated controls and normalized to housekeeping genes. On the other hand, lower concentration of nilotinib, 20 nM, increased/inhibited expression levels of 2/22 genes more than 2-fold comparing to untreated controls and normalized to housekeeping genes. Nilotinib induced expression levels of apoptotic (Bax, Serpin5B, GZMA, TNF, TNFRSF25, APAF1) cell cycle controlling (CDK2, CDKN2A, MDM2), and inhibitor of metastasis (TIMP1, TIMP3) genes and decreased expression levels of growth inducing (AKT1, IGF1, MYC, NFKB, MAP2K1, PLAU), antiapoptotic (SNCG, SYK), metastatic (MMP1, MMP2, ITGB5, ITGA3) and angiogenic (IL-8, ANGPT2) genes. The highest increases were observed in apoptotic TNF and GZMA genes while the highest decreases were observed in growth inducing MAP2K1 and PLAU genes. Summary/Conclusion. In this study, we demonstrated the molecular mechanisms of nilotinib induced cell death in addition to inhibition of oncogenic BCR/ABL protein. More importantly, we have also showed for the first time that nilotinib also has the potential to inhibit metastasis and angiogenesis through manipulating metastatic and angiogenic genes.

0725

IMMUNOGLOBULIN TREATMENT IMPROVES RECONSTITUTION OF B CELL COMPARTMENT AFTER AUTOLOGOUS STEM CELL TRANSPLANTATION

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Background. Early recovery of lymphocytes after stem cell transplantation is critical for a better survival and deficient B-cell functional recovery is due to decreased T-cell help and intrinsic B-cell defects. Studies after stem cell transplantation (SCT) demonstrated a delayed recovery of *in vivo* B-cell production of immunoglobulins. One way to improve reconstitution may be trough stimulation with molecules promoting lymphocyte development and function, as it is the case for natural antibodies. *Aims.* Evaluation of the effect of polyclonal immunoglobulin infusion or its fragments in promoting immune reconstitution after autologous stem cell transplantation (ASCT) in a lymphopenic *in vivo* model. *ethods.* Balb/c mice recipients, conditioned with lethal radiation, were distributed into four groups to test different weekly treatments: mouse polyclonal immunoglobulins (IgG), its derivatives Fab or Fc (250 μg/mouse/week), or saline (control). Evolution of peripheral immune reconstitution was monitored weekly up to 13 weeks (w) after ASCT. Age and sex-matched healthy Balb/c mice were used for normal references. Quantitation of B cells was performed by flow cytometry; serum levels of immunoglobulins and B-cell Activating Factor (BAFF) were measured by ELISA. Seric IgM reactivity against membrane protein extracts (*E. coli*) was assessed by quantitative immunoblot. Results were compared using SigmaPlot11.0 and IgorPro statistical softwares. *Results.* Recovery of B cell numbers was similar among groups. However, serum immunoglobulin recovering was significantly improved in treated groups. At 9w after ASCT, Ig-treated groups significantly increased IgM concentrations compared to control (P=0.002), most clearly for Fc and Fab treatments. Regarding recovery of serum IgG, all treated groups reconstituted better than control, at later time points. Fc-treated mice increased significantly comparing to control after 9w of follow up (FU) (P<0.001), surpassing normal values (n=10, md=695 ug/mL serum [25-75%]=[455-875]; maximum at 9-13w for Fc-group (n=6): 711 [466-1,188]; control (n=7): 143 [103-232]). All groups showed high BAFF serum levels at the first weeks of FU, not differing from normal (n=10, 7,419pg/mL [7,261-7,906]). During reconstitution, BAFF levels decreased in all groups similarly, being well below normal (P≤0.001) at the end of FU. Peripheral blood B cell counts and serum BAFF levels showed a significant negative correlation in Ig-treated mice (r=-0.845, P<0.001, 2-5w). At the first weeks, a positive correlation was also found between B cells and IgM (dominated by polyclonal-IgG: r=0.497, P=0.006) and B cells and IgG (dominated by Fab: r=0.511, P=0.013). Soon after ASCT, all groups exhibited low IgM reactivity, but treated groups showed a significantly faster recovery of IgM reactivity (OD measurements) after the 7th w comparing to control (polyclonal-IgG, n=12, P=0.0005; Fab, n=10, P=0.002; Fc, n=7, P=0.002), achieving significant higher values of IgM diversity compared to control. The control group seemed to recover only from the 10th w on. *Conclusions.* Our results suggest that treatment with polyclonal immunoglobulin (natural antibodies) during immunological recovery after ASCT promotes a better and more competent recovery of the B cell compartment.

0726

CLINICAL SIGNIFICANCE OF CLONALITY AND EPSTEIN-BARR VIRUS INFECTION IN ADULT PATIENTS WITH HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

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Background. In adults, hemophagocytic lymphohistiocytosis (HLH) is often associated with a variety of infections, malignant neoplasms, drugs, autoimmune diseases, and various immunodeficiencies. Unfortunately, it is difficult to make a diagnosis of lymphoma-associated HLH because fatal conditions delayed to perform the tissue biopsy and it took a long time to confirm diagnosis. A lack of histological proof of lymphoma can delay the choice of appropriate treatment for lymphoma-associated HLH at the initial stage. So, we hypothesized that early diagnosis may be supported by molecular study of the EBV genome and the clonal rearrangement of immunoglobulin (IG) and T cell receptor (TCR) genes. *Aimes*. We assessed the clinical significance of T or B cell clonality and Epstein-Barr virus (EBV) infection in adult patients with hemophagocytic lymphohistiocytosis (HLH) to identify factors related to prognosis. *Methods*. A total of 30 adult patients with diagnosed HLH were included in the study. In all patients, EBV-DNA in peripheral blood was examined by quantitative real-time polymerase chain reaction and bone marrow cells were examined for clonal rearrangement of T cell receptor gamma (TCRG) and immunoglobulin heavy chain (IGH) genes. Twenty of 30 cases were treated with systemic immunotherapy or immunochemotherapy and four cases were treated with combination chemotherapies of lymphoma regimen initially. Four patients received conservative therapy only, and 2 patients were transferred to other hospitals. *Results.* With a median follow-up of 4.6 months (range, 0.1-93.1 months), the median overall survival was 6.9 months and the estimated overall survival rate at 1 year was 47.6±9.7%. When we analyzed the factors affecting survival, none of the clinical features or laboratory findings was found to affect survival except for excess viral load of EBV-DNA. Patients with ≥ 1000/mL copies of EBV-DNA showed poor overall survival compared with those with low EBV-DNA load (P=0.010) (Figure 1).TCRG clones were detected in 10 patients (33.3%) and IGH clones were detected in 8 patients (26.7%). However, the clonality of TCRG(P=0.273) and IGH (P=0.182) did not have any prognostic role in survival rate at patients with HLH. *Conclusions.* Our results suggest that TCRG and IGH rearrangement do not have any clinical significance in adult patients with HLH but that high viral load of EBV-DNA may be a risk factor for poor outcomes. In HLH, high viral load of EBV-DNA should thus suggest a prompt approach with aggressive therapeutic interventions.

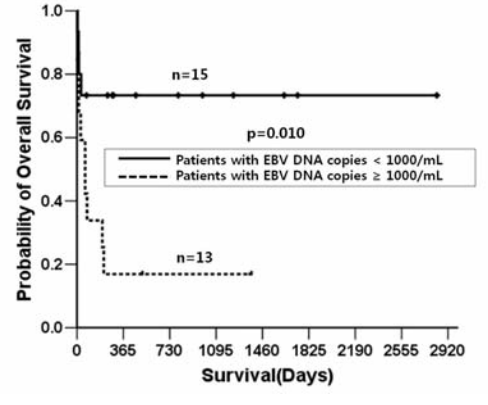

Figure 1. Overall survival according to EBV-DNA viral load.

0727

A NATIONWIDE SURVEY OF PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA IN FINLAND

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Background. Paroxysmal nocturnal haemoglobinuria (PNH) is an acquired syndrome characterised by intravascular hemolysis, venous thrombosis and bone marrow abnormality because of a deficiency of glycophosphoinositol-anchored (GPI) proteins from a subset of bone marrow-derived cells. The diagnosis is made by flow cytometry of GPI-anchored complement inhibitors CD59 and CD55 on blood cells. In Finland the diagnostics has been centralised to the Helsinki University Central Laboratory (HUSLAB) since 1992. *Aims.* Our aim is to conduct a nationwide survey on PNH in Finland. During a study still ongoing we have investigated the natural history of the disease, age and sex distribution, triggering factors, incidence, prevalence, co-morbidity, fate during pregnancy, therapy outcome, possible prognostic factors and life expectancy. A particular attention has been paid on spontaneous remissions, thrombotic events and the co-existence of aplastic anemia. *Methods.* As a starting point we have used laboratory databases of diagnosed cases. RBCs have been divided into three groups (I,II,III) based on the level of CD59 expression. Additional laboratory parameters included haemoglobin, haematocrit, lactate dehydrogenase, haptoglobin, erythrocytes, leucocytes, thrombocytes, inflammation markers (e.g. CRP), bone marrow analysis and urine haemoglobin. Clinical data was collected from patients' records and an additional questionnaire was filled in together with the patients' clinicians. This data included the symptoms and conditions attributable to PNH; haemoglobinuria, thrombotic events, infections, fatigue, renal failure, pulmonary hypertension, abdominal pain, dysphagia, dyspnoea, erectile dysfunction, anaemia and co-existence of aplastic anaemia, myelodysplastic syndrome and acute myeloid leukemia. All patients participated with written informed consent. *Results.* So far 71 patients, 38 female and 33 male, were been found from Finland with a population of 5.2 millions. From the cases studied the prevalence of PNH was calculated approximately as 1:100 000. The mean age at diagnosis is 44 years, range 5-76 years. Clinical data has until now been obtained from 28 patients. Nearly half of the patients were diagnosed also with aplastic anemia and about one third had had a thrombotic event. Abdominal pain had occurred in 21% of patients and 3/28 of the patients had Budd-Chiari syndrome. From 4 patients who underwent allogeneic stem cell transplantation 3 are in remission. Myelodysplastic syndrome, erectile dysfunction, kidney failure and spontaneous abortion each occurred in 2/28 patients. *Conclusions.* Further clinical data is being collected and placed in a nationwide registry to serve both academic research and clinical practice. Since PNH is a very rare disease, the results of this study will help both in the clinical decision making and standardization of the treatment as well as in the development of new therapies. The progress and prognosis of the disease are highly variable. Considering the rarity of the disease, centralised treatment should be considered.

0728

MECHANISMS INVOLVED IN PREMATURE SELF-DESTRUCTION OF RED BLOOD CELLS UNDER DIFFERENT ERYPTOSIS STIMULI

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Background. Recently, it has been reported that under certain circumstances erythrocytes (RBCs) can suffer premature self-destruction showing cell changes that mimic features of apoptosis in nucleated cells. Although much information about this process called eryptosis is available, the mechanisms are not yet well known. Aim. Based on the hypothesis that oxidative stress and increased intracellular calcium induce eryptosis through different pathways, the aim of this work was to study the mechanisms involved in this process under these two models of eryptosis. *Methods.* RBCs from healthy donors were obtained following informed consent. Cells were exposed to different eryptosis stimuli in buffer HEPES at 37°C for different periods. Morphological changes were observed by scanning electron microscopy. Flow cytometry assays were performed to detect Anexin V binding and to measure GSH, ROS, intracellular calcium, and band 3 levels. Membrane protein phosphorylation and nitrosylation were analyzed by SDS-PAGE and Western blotting. *Results.* The presence of either H_2O_2+$NaNO_2$ or Ca ionophore A23187 (CaI) induced membrane phosphatidylserine translocation (Control 1.8±0.6%; H_2O_2+$NaNO_2$ 71.6±27.3%; CaI 96.2±3.1%; H_2O_2+$NaNO_2$ *vs.* C P<0.05; CaI *vs.* C P<0.01, n=5). Interestingly, both treatments caused different morphological changes. Spherocytes with microvesiculation or stomatocytes were the characteristic cell shapes of CaI- or H_2O_2+$NaNO_2$-induced eryptosis, respectively. Significantly higher ROS and lower GSH levels with respect to untreated cells were observed in the presence of H_2O_2+$NaNO_2$ (P<0.05, n=3), whereas they remained unchanged under CaI exposure. In contrast with CaI treatment, no change in intracellular calcium levels was observed in the presence of H_2O_2+$NaNO_2$ (expressed as Gm of fluorescence intensity: C 53.3±6.7; H_2O_2+$NaNO_2$ 44.9±5.5; CaI 136.1±68.5; CaI *vs.* C P<0.01, n=4). Even though these results suggested that calpain activation may play an essential role to induce eryptosis, we did not detect a reduction of Anexin V positive cells in the presence of Calpain Inhibitor I under either H_2O_2+$NaNO_2$ or CaI incubation. Membrane protein analysis showed a strong reduction of band 3 after exposure to CaI (Flow cytometry, Gm: C 100.0±23.0; CaI 27.4±7.1; P<0.05, n=3) whereas post-translational modifications, such as nitrosylation of membrane proteins and band 3 phosphorylation were caused by cell incubation with H_2O_2+$NaNO_2$. Parallel assays to detect protein phosphorylation in cells pre-treated with staurosporine (kinase inhibitor) or sodium o-vanadate (phosphatase inhibitor) suggest higher involvement of kinase activation in eryptosis induced by H_2O_2+$NaNO_2$. *Conclusions.* RBCs exposed to oxidative stress or CaI suffered self-destruction detected by increase of Anexin V binding, although different pathways are involved in the mechanisms of eryptosis depending on the stimuli. On the one hand, an increase in calcium influx appears to specially affect membrane proteins such as band 3, possibly due to membrane vesiculation. On the other hand, oxidative stress not only reduces the amount of cellular protective anti-oxidative compounds, but also produces membrane protein modifications probably leading to alteration of their properties. Based on the present results we can suggest that the anemia observed under several diseases associated with chronic inflammation might be produced not only by erythroid progenitor dysfunction but also by direct effects of pro-eryptotic factors on erythrocytes.

Cellular immunotherapy and vaccination

0729

HIGH AVIDITY PRAME SPECIFIC T-CELLS DERIVED FROM A PATIENT AFTER HLA MISMATCHED STEM CELL TRANSPLANTATION, POTENTIALLY USEFUL FOR THERAPEUTIC TCR GENE TRANSFER STRATEGIES

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Adoptive immunotherapy with T-cells can effectively treat patients with hematological malignancies, EBV-associated malignancies and metastatic melanoma. Application of this therapy for these tumors is broadening by recent progress in genetic engineering of T-cells with genes encoding T cell receptors (TCR) specific for antigens expressed by these malignancies. TCRs with specificity for non-polymorphic tumor-associated self-antigens that are shared between various tumors are promising candidates. However, the isolation of high avidity T-cells that are specific for non-polymorphic tumor associated self-antigens is difficult, because of self-tolerance. During thymic selection T-cells exhibiting high avidity for self-antigens presented by self-HLA are deleted, and this most probably explains why most T-cells characterized to date directed against non-polymorphic tumor associated self-antigens exhibit low to intermediate avidity. After HLA mismatched stem cell transplantation, however, donor T-cells which have been educated in the donor have not encountered the allogeneic HLA molecules from the patient. Consequently, these T-cells can exhibit high avidity for tumor associated self-antigens presented by allogeneic patient specific HLA molecules. In this study we investigated whether high avidity T-cells directed against non-polymorphic tumor associated antigens restricted by allo-HLA could be selected from patients transplanted with HLA mismatched stem cell grafts. From one patient after HLA-A2 mismatched SCT at the time of graft *vs.* host disease we isolated T-cells specific for a peptide (SLL) of the tumor antigen PRAME (preferentially expressed antigen on melanomas) presented in HLA-A2. To determine whether the T-cells were solely PRAME-SLL peptide specific, T-cells were tested against T2 cells loaded with multiple HPLC fractions of peptides eluted from HLA-A2. The clone showed specific recognition of only one HPLC fraction, and by two additional HPLC fractionations and mass spectrometry we demonstrated that the SLL peptide was the only peptide that was recognized. In addition, by transfection of PRAME into HLA-A2 positive COS cells we demonstrated that endogenously processed PRAME was recognized. Peptide titration showed that the clone exhibited high avidity for the PRAME peptide (IC_{50}:50pM). HLA-A2 transduced K562 cells, known to highly express PRAME, were strongly recognized as well as a large fraction of tumors, including melanomas (90%), renal cell carcinomas (66%), non-small cell lung carcinomas (50%), and acute myeloid leukemia (AML) (50%). Since these T-cells exerted high avidity for PRAME we investigated whether HLA-A2+ non-malignant cells were recognized. The T-cells showed no reactivity against fibroblasts, keratinocytes, bronchus epithelial cells, hepatocytes, billiair duct epithelial cells, colon epithelial cells and mesenchymal stem cells. Also (activated) B-cells, (activated) T-cells, monocytes and $CD34^+$ cells were not recognized by the T-cells. However, the T-cells demonstrated reactivity against mature monocyte derived DC's and low reactivity against kidney epithelial cells. PRAME RNA expression levels of the tested target cells, determined by quantitative RT-PCR, demonstrated that the level of recognition correlated with PRAME expression. Based on these results we demonstrate single peptide specificity of allo-HLA reactive PRAME specific T-cells, high reactivity against numerous tumors and limited on-target toxicity. This PRAME specific TCR is a potential interesting tool for adoptive T-cell therapy using TCR gene modified T-cells.

0730

NATURALLY OCCURING HLA-G EXPRESSING CELLS IN PERIPHERAL BLOOD OF HEALTHY INDIVIDUALS WITH IMMUNOREGULATORY PROPERTIES

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Introduction. HLA-G is a nonclassic MHC class I with unique features that functions as an immunomodulatory molecule. HLA-G was initial-

ly found on trophoblasts, where it contributes to tolerance at the materno-fetal interface. HLA-G has been shown to regulate the rejection process after solid organ transplantation but its role in allogeneic hematopoietic cell transplantation (HCT) is unknown. Objectives. The aim of this study was to detect and characterize naturally occurring HLA-G$^+$ cells (HLA-Gnat) in peripheral blood and their possible immunomodulatory properties. Furthermore we investigated the effect of the demethylating agent 5-aza-2'-deoxycytidine (5-AC) on HLA-G expression and the generation of HLA-G induced cells (HLA-Gind). *Methods.* Peripheral blood mononuclear cells (PBMC) were obtained from 22 volunteer donors and were analyzed by FACS for the presence of HLAG$^+$ (MEM-G/9 MoAb) cells, both in whole PBMC and in specific cell subsets. Moreover, we isolated HLA-G$^+$ cells by FACS sorting (Vantage, BD) and analyzed their tolerogenic properties by using them as third party cells in mixed lymphocyte cultures (MLC). The lymphoproliferative response in the cultures was measured using the CFSE cell proliferation assay. The immunmodulatory effect of HLA-G was calculated in comparison to lymphocyte proliferation in control cultures without addition of HLA-G$^+$ cells. Furthermore, inhibition assays were performed with target cells that have been incubated with the anti-HLA-G1 87G moAb or an irrelevant isotypic control Ab. PBMCs and HLA-G neg cell lines (HaCaT, K562) were treated with 5-AC for 3 days, at final concentrations of 1 μM, 2.5 μM, 5 μM, 10 μM and 100 μM. HLA-G cell surface expression was determined with the MEM-G/09 mAb. *Results.* The mean percentage of HLA-G$^+$ cells in whole PBMC was 4.4% with a Specific Fluorescent Index (SFI) of 1.2. The mean percentage of HLA-G$^+$ cells was significantly higher in CD14$^+$ (23.2%) than in CD3$^+$ cells (1.4%), P=0.0002. Inversely, we isolated by FACS sorting HLA-G$^+$ cells from PBMC and found that 93% of them were CD14$^+$. CD14$^+$/HLAG$^+$ cells showed significant lower expression of HLA-DR (SFI 81.3) as compared to CD14$^+$/HLAG- cells (SFI 186), P<0.001. When FACS sorted CD14$^+$/HLAG$^+$ cells were added as third party cells in MLC we observed a dose dependent suppression of lymphoproliferation. Concentrations >1/1 of responder/HLA-G$^+$ cells showed significant suppression of the allo-response by 52% over the control, P=0.0012. The HLA-G mediated inhibition of lymphocyte proliferation could be reversed by masking HLA-G1 with blocking 87G moAb. HLA-G neg cell lines and CD3$^+$/HLAG- cells were found to express membrane HLA-G upon their *ex vivo* exposure to 5-aza-2'-deoxycytidine (HLA-Gind) in a dose dependent way (0.32% in the absence of 5-AC *vs.* 68% after treatment with100 μM 5-AC). *Conclusions.* Our results reveal the existence and the capacity to isolate HLA-G$^+$ cells with immunosuppressive function from PB of healthy individuals. Naturally occurring HLAG+ monocytic cells (and maybe also HLA-G$^+$ induced T-cells) suggest a promising strategy for adoptive cell immunosuppressive therapy after HCT.

0731

CYTOKINE INDUCED KILLER (CIK) CELLS FOR CELL THERAPY OF ACUTE MYELOID LEUKEMIA (AML): IMPROVEMENT OF IMMUNE ACTIVITY BY EXPRESSION OF CD33-SPECIFIC CHIMERIC RECEPTORS (CAR)

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Background. CIK cells are ex vivo expanded effector cells with potent antitumoral activity.We demonstrated that CIK cells' infusion in AML patients relapsing after allogeneic transplant is well tolerated, but limited clinical responses were observed. *Aims.* to improve anti-leukemic CIK functions. *Methods.* CIK cells were genetically modified with SFG-retroviral vectors coding for anti-CD33-zeta or anti-CD28-OX40-zeta CAR and their ability to kill leukemic targets was analysed either by 4h-51Cr-release assays and long-term co-culture on stromal cells. CD33-specific proliferation was assessed by 3H-thymidine incorporation and cytokine release by flow cytometry. *Results.* anti-CD33-zeta or anti-CD28-OX40-zeta CAR expressing CIK cells (average CAR expression, 65%) acquired potent cytotoxicity against several AML targets: after 4-hour incubation we observed, at effector: target (E:T) ratio of 5:1, a mean lysis of the HL-60 cell line of 79% and 75%, for anti-CD33-zeta and anti-CD33-CD28-OX40-zeta cells respectively (P<0.005 *vs.* untransduced CIK cells). Analogous lytic efficiency was registered against the KG-1 cell line, known to be resistant to Gentuzumab-Ozogamicin, and primary AML blasts, with cytotoxicity >50%, contrarily from non transduced CIK cells, that showed <10% lysis against both targets (P<0.005). The strong anti-leukemic activity of manipulated CIK cells was confirmed in long-term killing experiments, where leukemic cells were co-coltured with CIK cells for 6 days on human stromal layer without exogenous IL-2 at an E:T ratio of 1:200.In these assays, anti-CD33-CD28-OX40-zeta CIK cells eliminated almost all leukemic cells, with 16% mean residual primary AML cells compared to 91% (P≤0.005) of untransduced CIK cells. Anti-CD33-zeta CIK cells showed analogous but lower efficiency, with 31% mean residual primary AML (P≤0.05). Moreover, a prominent CD33-specific proliferative activity was observed, with a mean proliferation index of 2.2 after HL-60-mediated stimulation and 2.4 after primary AML cells-mediated stimulation in anti-CD33-zeta transduced cells compared to 0.9 (P≤0.005) and 1.4 (P≤0.05) for un-transduced CIK cells. Expression of the anti-CD33-CD28-OX40-zeta CAR on CIK cells resulted in a higher expansion rate, with a mean proliferation index of 4.4 (P≤0.005) after HL-60-mediated stimulation and 3.7 (P≤0.005) after primary AML cells-mediated stimulation. In addition, when stimulated with irradiated HL-60, anti-CD33-zeta and anti-CD33-CD28OX40-zeta CAR-transduced CIK cells secreted 11-fold and 10-fold higher amount of IFN-gamma (P≤0.005 for anti-CD33-zeta and anti-CD33-CD28-OX40-zeta), 120-fold and 180-fold more TNF-alfa (P≤0.005 for anti-CD33-zeta and for anti-CD33-CD28-OX40-zeta), 250-fold and 600-fold more TNF-beta (P≤0.005 for anti-CD33-zeta and for anti-CD33-CD28-OX40-zeta), 1400-fold and 3800-fold more IL-2 (P≤0.05 for anti-CD33-zeta and for anti-CD33-CD28-OX40-zeta) compared to unmanipulated cells. Importantly, anti-CD33-CAR expressing CIK cells showed a transitory toxicity against normal hematopoietic CD34$^+$ progenitors, with a reduced, but consistent number of clonogenic progenitors recovered in colony forming-assays after up to 48-hour co-culture with transduced CIK cells. *Conclusions.*our results indicate that anti-CD33 CARs strongly enhances anti-leukemic CIK functions, suggesting that CAR-expressing CIK cells might represent a promising tool for AML immunotherapy.

0732

CD137 STIMULATION ENHANCES ANTI-LYMPHOMA ACTIVITY OF RITUXIMAB

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Background and Aims. Rituximab, a monoclonal antibody (mAb) directed against CD20, has significantly improved the outcome of patients with lymphoma. Antibody-dependent cell-mediated cytotoxicity (ADCC), largely mediated by natural killer (NK) cells, is thought to play an important role in rituximab's efficacy. In the current study, we hypothesized that the anti-lymphoma activity of rituximab could be enhanced by stimulation of NK cells with an anti-CD137 agonistic mAb. CD137 (4-1BB) is a costimulatory molecule expressed on a variety of activated immune cells, including T and NK cells.

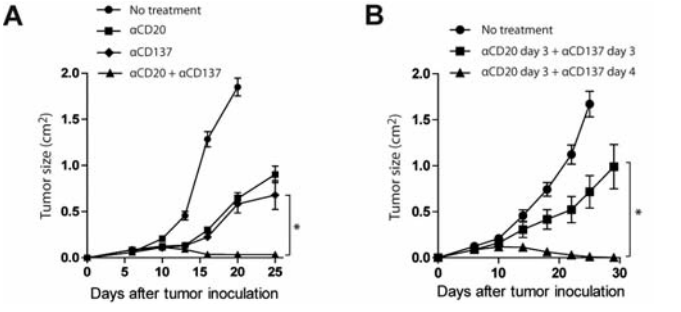

Figure 1.

Methods and Results. Using human primary lymphoma samples, we found that, while resting human NK cells do not express CD137 at baseline, these cells highly upregulate CD137 when encountering rituximab-coated tumor B cells. Furthermore, anti-CD137 agonistic mAb significantly enhances rituximab-induced NK cell degranulation and cytotoxicity as measured by CD107a mobilization (P=.006) and chromium release (P=.01). In a syngenic murine lymphoma model, anti-CD137 mAb significantly enhances anti-tumor activity of anti-CD20 mAb leading to complete tumor resolution (Figure 1A, P<.001) and prolonged survival (P=.048). This synergistic effect is dependent upon sequential administration first of anti-CD20 mAb and then followed by anti-CD137 mAb (Figure 1B, P<.001). We show that in-vivo administration

of anti-CD20 mAb induces upregulation of CD137 on mouse NK cells (P=0.001) after 24 hours, allowing their activity to be enhanced by the anti-CD137 mAb. Consistent with this observation, NK cell depletion completely abrogates the therapeutic effect of anti-CD20 plus anti-CD137 mAb combination (P<.001). Conclusions. Our study presents a novel, sequential antibody approach to enhance rituximab's efficacy in patients with B cell malignancies by selective stimulation of rituximab-activated NK cells with anti-CD137 mAb. These results support the evaluation of anti-CD137 mAb in combination with rituximab in clinical trials for patients with lymphoma.

0733

REDIRECTING T-CELLS AGAINST CANCER CELLS BY TRANSFER OF BROADLY TUMOR REACTIVE GAMMA-DELTA-T-CELL RECEPTORS

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Background. Adoptive transfer of alpha-beta-T-lymphocytes is a promising treatment for a variety of malignancies, but often not feasible due to difficulties in generating T-cells reactive with the targeted antigen from patients. To facilitate rapid generation of cells for therapy, T-cells can be programmed with genes encoding for a tumor-specific high-affinity gamma-delta -T-cell receptor (TCR). Selective anti-tumor-reactivity and ignorance of the healthy-environment by gamma9-delta2-T cells arises from its ability to recognize non-classical antigens, including the non-peptidic intermediates of isoprenoid biosynthesis and so called stress signals on the tumor cells. *Aims* and *Methods.* To test the ability of gamma9-delta2-TCRs to redirect alpha-beta-T cells selectively against tumor cells, the gamma9-delta2-TCR was retrovirally transduced into human alpha-beta T cells. *Results.* Thereby, strong surface-expression of introduced gamma9-delta2-TCRs was observed while endogenous alpha-beta TCR chains were down-modulated. Functional analysis revealed that a gamma9-delta2-TCR efficiently reprograms both, $CD4^+$ and $CD8^+$ T-cells against a broad panel of cancer cells while ignoring normal cells. Moreover, tumor-specific T-cell proliferation, cytokine secretion and killing were significantly enhanced by additional application of biphosphonates. Finally, gamma9-delta2-TCR transduced T cells reduced colony-formation of progenitor cells of primary acute myeloid leukemia blasts. Recognition depended primarily on the presence of phospho-antigens. Expression of NKG2D ligands and F1-ATPase contributed to the activity of gamma9-delta2-TCR transduced T-cells but were not mandatory. Summary. Gamma9-delta2-TCRs are an attractive alternative to redirect alpha-beta T cells against cancer cells with both, an improved efficacy and safety profile as compared to currently used TCRs.

0734

PRAME RECOMBINANT PROTEIN VACCINATION CAUSES SIGNIFICANT TUMOR REDUCTION IN THE ANIMAL MODEL

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Background. The PRAME (preferentially expressed antigen of melanoma) has been earlier discovered as a naturally occurred target for the cytotoxic $CD8^+$ T-cells in some of the melanoma patients with atypically moderate clinical course of this malignant disease. It is also shown that PRAME gene is overexpressed in the vast variety of the human malignances including not only melanoma and any other solid tumors but also acute and chronic leukemias and lymphomas. There are promising data that PRAME-derived peptides are able to render anti-tumor cytotoxic $CD8^+$ T-cell-based effect being used as a substance for the vaccination. At least one of the molecular functions of the PRAME protein is to inhibit nuclear retinoic acid receptor alpha (RARα). This feature contributes to and aggravates the tumor malignancy. The importance of the PRAME expression is stressed by the fact that it is the main one that is activated in terminal phase of the chronic myeloid leukemia. The PRAME is considered to be a useful target for developing of the anti-tumor approaches based on the immunotherapy techniques as well as on the search for the low-weight inhibitory compounds. *Aim.* The main aim of this study was to estimate the value of the whole-sized human recombinant protein as an anti-tumor vaccine against PRAME-expressing tumor cells. *Methods.* We have cloned a coding sequence of the human PRAME gene in the pET32a expressing vector. The recombinant PRAME protein carrying 6-His tag has been further purified by means of the Ni-agarose column. Prior to purification we have developed conditions to refold recPRAME out of insoluble inclusive bodies. The final recPRAME was of high purity and occurred to be extremely stable in the form of the aqueous sterile 2 mg/mL solution being stored at +4C. The PRAME coding sequence was also cloned in the eukaryotic expressing vector pCEP (Invitorgene) under the strong CMV promoter. All mice were F1 female hybrids Balb/C*DBA2. Mouse SP2/0 multiple myeloma and B16-F1 melanoma cell lines have been transfected by PRAME/pCEP and constantly PRAME expressing cell lines have been selected. In every round of immunization we have used 35 animals. First mice set were vaccinated into abdomen 4 times every 2 weeks by the dose of 100 micrograms of recPRAME supplemented by 300 micrograms of aluminum hydroxide adjuvant (Sigma). The other 35 mice were inoculated with the only Al hydroxide as a negative control. We have also used an equal group of mice without immunizations as the other type of the negative control. 400000 PRAME/pCEP-transfected cells and 400000 cells transfected with the pCEP vector without PRAME insertion were engrafted into upper dorsal part of every mouse. For every cell types we have used the following sets of mice: 15 mice immunized by recPRAME, 15 mice inoculated by only Al hydroxide, and 15 mice without any immunization steps. The extra 5 mice from every group of animals were used for blood counts, ELISA estimation of the anti-PRAME antibodies and in the anit-tumor leukocyte cytotoxicity tests. *Results.* We have demonstrated that recPRAME is not toxic taking into account insignificant difference in blood values among experimental and control groups of animals and integrity of animal tissues analyzed by immunohystochemistry. Immunization by recPRAME created a strong and significant humoral response which has been evaluated by high presence of both IgM and IgG anti-PRAME antibodies in the recPRAME immunized animals. The clear anti-tumor effect of the recPRAME vaccination was demonstrated by the 3 time PRAME-positive tumor growth suppression as compared with the growth of the PRAME-negative tumor cells in the case of myeloma. This effect in the case of the melanoma cells was even more significant due to 5 time PRAME-positive tumor cell growth reduction. We have also used monoclonal antibodies against PRAME obtained in our lab to characterize PRAME protein cellular localization. All transfected cells as well as K562 cells and human melanoma cell lines (a kind gift of Prof. A. Baryshnikov) shows a distinct staining of the cellular membrane surfice. *Conclusion.* Our data confirms previously mentioned anti-tumor effects of the PRAME vaccination. In our study we further extend these observations and have shown that not only PRAME-derived peptides but the full-length recPRAME may be used in the vaccination against PRAME-positive tumors. It is especially important considering necessity to overcome HLA-restriction. The whole-sized recPRAME may potentially cover all HLA isotypes and be an invariant vaccine for every patient who suffers from PRAME- positive malignancy. The localization of the PRAME protein on the tumor cell surfice raised a possibility for the usage of the anti-PRAME antibodies as a therapeutic agent.

0735

THE DNA DEMETHYLATING AGENT 5-AZA-2'-DEOXYCYTIDINE INDUCES EXPRESSION OF NY-ESO-1 AND OTHER CANCER/TESTIS ANTIGENS IN MYELOID LEUKEMIA CELLS

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Background. Azanucleoside DNA-hypomethylating agents have remarkable clinical activity in myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML), particularly at low, non-cytotoxic doses. They are able to reactivate epigenetically silenced genes including, among others, a number of immunogenic proteins called cancer/testis antigens (CTAs). Germ cell restricted expression of CTAs in normal tissues and aberrant expression in various human cancers make them attractive targets for cancer immunotherapy. In contrast to solid tumors, CTA expression in myeloid malignancies is usually very low or absent. *Aim.* We aimed to reactivate epigenetically silenced CTAs using 5-aza-2´-deoxycytidine (DAC) and thereby trigger an immune response in various myeloid leukemia cell lines. *Methods.* Myeloid cell lines Kasumi-1, U937, THP-1 and HL-60 were treated with 5-aza-2´-deoxycytidine and mRNA expression of the CTAs NY-ESO-1, MAGEA1, MAGEA3 and MAGEB2 was quantified at days 2, 3, 6, 14 and untreated control using real-time RT-PCR. In addition, microarray expression analysis was carried out in DAC-treated Kasumi-1 cells. Expression of NY-ESO-1 was confirmed at the protein level. Recognition and functional activity of *de novo* expressed NY-ESO-1 in DAC-treated U937 cells was evaluated by IFN-g Elispot and standard 51chromium release

assay using the HLA-B51 restricted NY-ESO-1 p94-102 -specific T cell clone. DNA methylation of the NY-ESO-1 promoter region was analyzed by pyrosequencing. *Results.* Consistent time- and dose-dependent reactivation of all 4 CTA genes was observed, with maximum mRNA levels 72-144 h after treatment start. *De novo* induction of NY-ESO-1 correlated with DNA hypomethylation of the promoter region. As determined by RNA microarray analyses, numerous other CTA genes (located on the X-chromosome) were also derepressed in a time-dependent fashion by DAC. By Elispot and chromium release assays we showed that the *de novo* expressed protein was naturally processed and presented in a time- and dose-dependent fashion up to 8 days after the start of DAC treatment, and converted the cell lines susceptible to antigen-specific recognition by CD8+ T-cell clones. *Conclusions.* NY-ESO-1 and numerous other CTAs localized on the X-chromosome are readily and transiently reactivated in myeloid leukemia cell lines treated with DAC. The susceptibility of DAC-treated AML cell lines to antigen-specific T-cell recognition has clear implications for future clinical trials combining epigenetic therapy and specific immunotherapy in myeloid neoplasia.

0736

NATURAL KILLER T CELL ENGAGEMENT ENHANCES IMMUNOGENICITY OF CHRONIC LYMPHOCYTIC LEUKAEMIA CELLS

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Background. CLL is the most common leukaemia in Europe, and may be an attractive target for adoptive immunotherapy: Although CLL can be cured with allogeneic stem cell transplantation, this treatment is not an option for the majority of patients due to advanced age or co-morbidities. Invariant natural killer T (iNKT) cells recognise glycolipid agonists such as alpha galactosylceramide (aGalCer) presented by CD1d, rapidly producing large amounts of cytokines and shaping the subsequent immune response. aGalCer is a highly effective adjuvant to tumour vaccination in some murine models of cancer. *Aims.* We sought to assess the iNKT cell/CD1d axis in patients with chronic lymphocytic leukaemia (CLL) with a view to using aGalCer as an adjuvant in CLL immunotherapy. *Methods.* Following informed consent, peripheral blood mononuclear cells were isolated from 30 patients with untreated CLL and 30 healthy, age-matched controls. iNKT cells were enumerated and phenotyped by flow cytometry. *In vitro* iNKT cell proliferation in the presence of aGalCer and interleukin 2 was assessed, and iNKT cell lines generated from patients and controls. Chronic lymphocytic leukaemia cells were purified by flow cytometric cell sorting, pulsed with aGalCer or vehicle, and used as stimulators in mixed lymphocyte reactions. *Results.* There was a trend towards lower iNKT cell numbers in patients with CLL (median 0.01% of $CD3^+$ lymphocytes *vs.* 0.02% in controls). iNKT cells could be expanded from all controls and from 12 out of 14 patients with CLL, although fold expansion was lower in patients than controls. iNKT cell lines derived from patients and controls had similar cytokine producing capacity and lysed CD1d+ target cells in the presence of aGalCer. CLL cells themselves expressed CD1d, and pulsing CLL cells with aGalCer enhanced the subsequent mixed lymphocyte reaction against the leukaemic cells. *Summary/Conclusions.* The iNKT cell/CD1d axis is largely intact in patients with CLL, and functional iNKT cell lines can be derived from patients. Pulsing CLL cells with aGalCer enhances the subsequent mixed lymphocyte reaction. aGalCer pulsed tumour cells may prove an effective tumour vaccine in CLL.

0737

SERUM IL-7 AND IL-15 KINETICS DRIVE LYMPHOPENIA-INDUCED PROLIFERATION AFTER HIGH-DOSE CHEMOTHERAPY AND AUTOLOGOUS STEM CELL TRANSPLANTATION

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Introduction. Murine models have shown that interleukin (IL)-7 and IL-15 are required for the 2-step recovery of the absolute lymphocyte count (ALC) after high-dose chemotherapy, a phenomenon termed "lymphopenia-induced proliferation (LIP)". No human study to date has ever validated preclinical models. Aim. We studied kinetics of serum IL-7 and IL-15 levels during high-dose chemotherapy supported by autologous hematopoietic stem cell transplantation (HSCT) in patients with multiple myeloma (MM), Hodgkin's lymphoma (HL) or non-Hodgkin lymphoma (NHL). *Methods.* Informed consent was collected from 60 MM (Mel100 or Mel200), 15 NHL (BEAM) and 12 HL (BEAM) patients undergoing autologous HSCT. Blood samples were collected at admission, and on days +3, +6, +10, +40, +70, +100 after HSCT. Plasma IL-7 and IL-15 levels were measured by high sensitivity (> 0.1 pg/mL) Quantikine ELISA kits (R&D Systems, Minneapolis, MN) according to manufacturer's instructions. *Results.* Serum IL-7 and IL-15 levels inversely correlated with the ALC ($P < 0.001$). Serum IL-7 peaked at day 6 in BEAM and on day 10 in Mel100 and Mel200. Serum IL-15 peaked on day 6 in Mel200 and BEAM and on day 10 in Mel100 (Figure 1). The degree of the peak directly correlated with the dose of melphalan administered during conditioning (100 mg/m^2 < 200 mg/m^2 < BEAM). *Conclusions.* The differential day of peak of serum IL-7 and IL-15 is concordant with what is known from murine models about LIP. LIP is a dose-dependent phenomenon which in our model correlated linearly with increasing doses of melphalan. These results have implications for adoptive immunotherapy in the setting of high-dose chemotherapy.

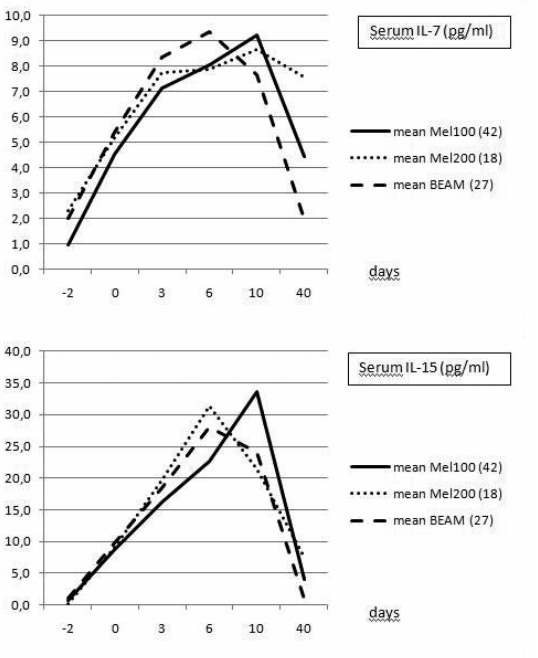

Figure 1. Serum levels of IL-7 and IL-15 according to differ.

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IMMUNE- MODULATION OF CD4+ AND CD8+ - CELLS BY 5-AZACYTIDINE

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Background. Demethylating agents like 5-Azacytidine (5-Aza) were introduced for treatment of myeloid dysplastic syndrome (MDS) and acute myeloid leukaemia (AML). Besides induction of differentiation, an additional suggested mechanism for 5-Aza is demethylation of antigens genes such as Cancer-Testis Antigens (CTA) which induce immune response by immunocompetent cells. Despite our extended knowledge on its effect on myeloid cells, 5-Aza impact on immune cells has not been yet clarified. Therefore, we investigated the effects of 5-Aza on T cells. *Materials and methods.* T-cells were isolated from buffy coats by magnetic cell sorting. Cells were stimulated and cultured for 1 week in the presence of IL-2. Thereafter, cells were treated with (5 μM or 20 μM) or without 5-Aza for 48h. mRNA was isolated and used for cDNA synthesis. qPCR was performed under standard conditions for expression of IL-10, FoxP3, and TGF-beta, normalized to GAPDH. T cells were analyzed by flow cytometry using the following antibodies: CD3, CD4, CD8, HLA-DR, FoxP3, CD127, CCR7, CD45RA, CD69, CD62L and CD25. Functional cytotoxicity of an AML cell line (HL60) was measured using a LDH release assay. *Results.* CD3 expression was not altered by 5-Aza treatment, but changes of T cell subpopulation subsets were observed: percentage of $CD8^+$ T cells decreased from 46% to 35%, whereas percentage of $CD4^+$ T cells increased from 57% to 68%. $CD3^+$ cells treated with none, 5 μM and 20 μM 5-Aza were stained for HLA-DR activation marker and exhibited reduced expression of 57%, 48% and 44% respectively, while mRNA level screening of IL10, FoxP3, and TGF-beta indicated upregulation (P=0.02, P=0.003 and P=0.02). Strikingly, $CD4^+FoxP3^+$ Treg cell counts elevated from 5% to 10% and 11% under aforementioned conditions. FoxP3 mRNA levels were also increased in $CD8^+$ cell compartment (P=0.01). In accordance with mRNA data, the different drug concentrations induced upregulation of $CD8^+FoxP3^+$ cells in a climax of 32 to 45%. Furthermore, we observed a persistence of a naive phenotype as indicated by $CCR7^+CD45RA^+$ markers in both T cell subpopulations. Interestingly, this effect was greater in the $CD8^+$ population. In a cytotoxicity assay we observed a significantly reduced capability of Aza- treated $CD3^+$ cells to lyse target cells (P=0.02). *Conclusions.* Our data indicate that 5-Aza induces FoxP3 expression and a regulatory phenotype in $CD4^+$ and $CD8^+$ cells. 5-Aza treatment supports inhibitory phenotypes by induction of inhibitory cytokines like IL10, while killing capacity of Aza treated cells is reduced. Moreover, naive T cells seem to be more stable during 5-Aza treatment. Therefore, we conclude that 5-Aza treatment constrains distribution and function of immune cells.

0739

IMPACT OF KIR-LIGAND AND KIR-GENE EXPRESSION ON NK CELLS MEDIATED KILLING OF MYELOMA CELLS

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Background. Natural Killer cells (NK cells) have the ability to recognize and kill malignant cells, like myeloma cells. Their killing ability is regulated by different groups of receptors that either activate the NK cell or inhibit its cytotoxic function. Killer Immunoglobulin like Receptors (KIR, CD158) are a family of molecules found on the surface of NK cells, estimated to inhibit their cytotoxic function. Both KIR2DL1 and KIR2DL3 receptors recognize the HLA-C2 and -C1 molecules respectively, when found on the corresponding cell. Both receptors trigger inhibitory pathways to protect tissues of NK mediated killing. We evaluated the impact of the KIR2DL1/2DL3 on the cytotoxicity of NK cells against different myeloma cell lines which express different HLA-C molecules. *Material and methods.* Three different myeloma cell lines (KMS12BM [C1/C1], MOLP8 [C1/C2] and RPMI8266 [C2/C1]) and a NK cell line (NKL) were cultured under standard conditions. NKL cells were transfected with cDNA for human KIR2DL1 and KIR2DL3, respectively. For RNAi experiments two siRNA and two control siRNA were used. NK cells from healthy donors were isolated by magnetic cell sorting. Thereafter NK cells were transiently transfected with the siRNA or control siRNA for up to 48 h. NK cell cytotoxicity was measured using a LDH release assay. *Results.* This study investigates whether the HLA-C genotype is adequate for protecting myeloma cells of NK mediated cell death. When NK cells, that are genotyped for HLA-C1/C1, were co-cultured with different myeloma cell lines, C1/C1 myeloma cells were rescued in contrast to myeloma cell lines that express C2/C1 (12% cytolyses *vs.* 38% and 45% cytolyses, respectively, P<0.05). KIR receptors, by recognition of HLA-C molecules, are believed to reduce NK cell cytotoxicity. We used NK cells from healthy donors, which express KIR2DL1 (recognizing C2) to evaluate whether receptors´RNAi knock down could increase the ability of NK cells to lyse target cells. Knock down of KIR2DL1 in NK cells results in a highly efficient killing of a C2/C2 target cell line (RNAi treated NK cells showed a cytotoxicity of up to 70%, P<0.05). To evaluate whether KIR receptors are major inhibitory molecules, we used NK cell line (wt NKL), a non expressor of KIR receptors that exhibited high cytotoxic potential against all tested target cell lines (>65% cytotoxicity). This NK cell line was transfected with KIR2DL1 or KIR2DL3 (recognizing C2, or C1). C2 positive myeloma cell lines were significantly rescued when exposed to KIR2DL1 transfected NK cells (wt NKL 72% *vs.* KIR2DL1 NKL 29%, P<0.05). Similar results were obtained when C1 positive target cell line was co- cultured with KIR2DL3 transfected NK cells. RNAi mediated silencing of KIR overexpression restores protection observed. *Conclusion.* We have shown that: 1) genotype recognition is sufficient for inhibition of NK cells dependent cytotoxicity, 2) KIR receptors are important inhibitory molecules for NK mediated cell death, while their down regulation highly affects their killing capacity. Therefore, KIR receptors emerge as an attractive target molecule for modulation of NK cell myeloma surveillance.

Chronic lymphocytic leukemia - Biology 2

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IDENTIFICATION OF RHAMM-DERIVED CD8+ RESTRICTED, HETEROCLITICAL, CRYPTIC EPITOPE R9Y AS A PROMISING TARGET FOR IMMUNOTHERAPY OF CHRONIC LYMPHOCYTIC LEUKEMIA

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The receptor for hyaluronic acid mediated motility (RHAMM) is leukemia associated antigen (LAA) strongly correlated with proliferation and bearing negative prognosis for chronic lymphocytic leukemia (CLL). Recently, we completed a peptide vaccination clinical trial with dominant RHAMM-derived epitope (R3) for patients with CLL. To our surprise, R3 vaccination induced regulatory T cells (Treg) in 66% vaccinated patients which might limit the clinical efficacy of peptide immunotherapy. Obtained results also pointed to a pre-existing tolerance to LAA-derived peptides. While the tolerance is limited to dominant epitopes we aimed to identify a crypitic epitopes that might circumvent tolerance mechanisms and in order to increase affinity modified them heteroclitically. We screened in silico 50 peptides with different heteroclitical modifications using SYFPEITHI algorithm and calculate affinity to MHC-I complex. Further, we choose 10 peptide pairs (cryptic - modified) which affinity increased to the highest extent and screened them *in vitro* assay for the affinity as well as the peptide-MHC-I complex stability in T2 binding test. In functional studies we screened 5 peptide pairs by ELISPOT analysis using $CD8^+$ T cells isolated from peripheral blood (PB) of CLL patients and healthy donors. We found that $CD8^+$ cells from CLL patients presensitized with peptides: R9Y (YLQLDAFEV); R13YL (YLVQSLEDV); R15Y (YLKQTLDEL); R17YL (YLQEQLNKI) as well as R19YL (YLIKHVVKL) specifically recognized T2 cells pulsed with respective peptides with the highest frequency for R9Y (90%). In chromium-51 release assays, R9Y-primed $CD8^+$ T cells from CLL patients were able to effectively lyse R9Y-peptide pulsed T2 cells as well as CLL leukemic cells presenting RHAMM in a HLA-A2 restricted manner; in contrast, K562 cells and T2 cells lacking either RHAMM or HLA-A2 expression were not lysed above background levels. In addition, mAb-based MHC I blockade demonstrated that the observed peptide-specific lysis was MHC I-restricted. In conclusion, we defined a novel RHAMM-derived CD8+ restricted, heteroclitical, cryptic epitope R9Y which might represent an interesting target for immunotherapy of CLL. In future clinical administration the R9Y peptide might circumvent tolerance to dominant epitopes and thereby increase efficacy of peptide vaccination.

0741

STUDY OF THE QUANTITATIVE, FUNCTIONAL AND IMMUNOREGULATORY PROPERTIES OF BONE MARROW MESENCHYMAL STEM CELLS IN PATIENTS WITH B-CELL CHRONIC LYMPHOCYTIC LEUKAEMIA

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Background. Leukemic B-lymphocytes in patients with B-cell chronic lymphocytic leukaemia (B-CLL) accumulate in the bone marrow (BM) possibly under the influence of locally produced nourishing/survival/homing signals. The possible involvement of BM mesenchymal stem cells (MSCs), representing the progenitors of the BM microenvironment cells, in the pathophysiology of B-CLL has not been extensively studied. *Aims.* To investigate whether the BM MSCs from B-CLL patients are primarily or secondarily defective by evaluating their quantitative/qualitative characteristics and immunoregulatory properties. *Methods.* BM aspirates from nine B-CLL patients (Binet stage A: 4; Binet stage B: 2; Binet stage C:3) and nine healthy donors were appropriately culture expanded to obtain the MSCs. The immunophenotypic (CD45/CD14/CD34 negative and CD70/CD90/CD105/CD44/CD29/CD73/CD146 positive) and survival characteristics of MSCs were evaluated by flow-cytometry. A colony-forming unit fibroblast (CFU-F) assay was used for the estimation of MSC frequency within the BM mononuclear cell (BMMC) fraction and the population doubling time for the evaluation of their proliferative capacity through passages. MSC differentiation capacity toward the osteogenic and adipogenic lineages was studied using appropriate culture conditions. Patients' MSC immunosuppressive function was evaluated by estimating the proliferation (^{3}H-thymidine method) of mitogen-induced (PHA 2 μg/mL or IL-2 500 Iu/mL) allogeneic normal $CD3^+$ cells in the presence of different concentrations of MSCs from patients or healthy controls. FISH analysis was also performed in MSCs cultures from passage-2 for possible detection of the genetic aberrations identified in patient haemopoietic cells (LSI ATM [11q22.3], CEP12, D13S319 [13q14.3], IGH [14q32.3], TP53 [17p13.1]). *Results.* MSC frequency was decreased in B-CLL patients (5.2±6.17 CFU-F per 10^5 BMMCs) compared to controls (15.32±17.41 CFU-F per 105 BMMCs; $P<0.05$) apparently due to BM leukemic infiltration. The immunophenotypic characteristics and differentiation potential of MSCs towards adipogenic (Oil red O stain and aP2 and PPARG mRNA expression) and osteogenic (ALP/Von Kossa stain and ALP and RUNX2 mRNA expression) lineages did not differ between patients and controls. The proliferative potential of MSCs through passages was reduced in patients compared to controls ($P<0.001$) and MSC cultures from 3 patients could not expand beyond passage-4. A statistically significant increased proportion of apoptotic cells (7-amino-actinomycin-D positive) was also observed in MSC cultures from patients compared to controls through passages ($P=0.0206$). MSC immunosuppressive function in terms of inhibition of mitogen-induced T-cell proliferation did not differ significantly between patient and normal MSCs. FISH analysis of MSC cultures did not identify the genetic aberrations observed in the respective haemopoietic cells in any patient. Summary/*Conclusions.* The BM MSCs from B-CLL patients display normal immunophenotypic characteristics and differentiation potential. The immunosuppressive properties of patient MSCs are normal suggesting that MSCs is unlikely to have a role in the pathogenesis of autoimmune complications associated with B-CLL. Patient MSCs are devoid of the genetic aberrations associated with the disease. MSCs however, display impaired proliferative potential and survival during culture expansion probably due to their previous exposure in the leukemic BM environment. The production of cytokines that may have a role in the survival, growth and homing of the malignant cells by patient MSCs is under investigation.

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MDM4/MDMX IS OVEREXPRESSED IN CHRONIC LYMPHOCYTIC LEUKAEMIA (CLL) AND MARKS A SUBSET OF P53WILD-TYPE CLL WITH A POOR CYTOTOXIC RESPONSE TO NUTLIN-3

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Background. p53 plays a key role in determining the clinical features of chronic lymphocytic leukemia (CLL). Disruption of p53 by point mutations and/or deletion at 17p13 occurs in a fraction of cases at diagnosis and predicts poor survival and chemorefractoriness. In cells with functional p53, p53 activity is inhibited through interaction with MDM2. In this regard, p53 can be activated upon exposure of cells to inhibitors of such interaction, like Nutlins. In fact, exposure of CLL cells to Nutlin-3 is effective in raising the levels of p53 protein with subsequent induction of cell cycle arrest and/or apoptosis independently of the most relevant prognostic markers. However, cells of a minority of p53 wild-type ($p53^{wt}$) CLL cases do not respond to Nutlin-3 treatment and fail to display the canonical signature associated with in-vitro Nutlin-3 exposure. *Aims.* to identify and validate the relevant genes associated with resistance of $p53^{wt}$ CLL cells to the in-vitro Nutlin-3 effects. *Methods.* purified cells from sixteen peripheral blood (PB) CLL samples, all with $p53^{wt}$ gene, were exposed to 10 microM Nutlin-3 for 24 hours. Gene Expression Profile (GEP) was performed using a dual labelling strategy; all validation experiments were performed by quantitative real-time PCR (qRT-PCR). *Results.* i) signature of Nutlin-3 exposure in $p53^{wt}$ CLL: 144 differentially expressed genes (143 up-regulated, 1 down-regulated) were correlated with the response to Nutlin-3. Among up-regulated genes, several genes were related to apoptosis (e.g. BAX, BBC3, IKIP, FAS, TRIAP1, GADD45, TP53INP1, ISG20L1, ZMAT3, TNFRS10C, TNFRSF10B/TRAIL-R2), while other genes were over-expressed upon ex vivo exposure to Nutlin-3 (e.g. MDM2, CDKN1A, PCNA) as a part of a negative feed-back mechanism. Of note, this signature was not shared by 3/16 $p53^{wt}$ cases ("non-responder"$p53^{wt}$ CLL); consistently, cells from these cases were significantly resistant to the in-

vitro cytotoxic effects of Nutlin-3; ii) signature of Nutlin-3 "non-responder"p53^{wt} CLL: comparing the constitutive GEP of 13 "responder"versus 3 "non-responder"p53^{wt} CLL, we obtained 278 differentially expressed genes, 149 up-regulated and 129 down-regulated in "non-responder"p53wt CLL. Among up-regulated genes, we focused on MDM4/MDMX, a gene whose product was known to have an inhibitor activity of p53-dependent transcription and to form Nutlin-3 resistant complexes with p53. iii) MDM4/MDMX expression in CLL: taken together, higher expression levels of MDM4/MDMX were detected in a different series of 43 CLL samples when compared to normal B cells of PB samples from 15 healthy donors (P<0.0001). Moreover, no differences in MDM4/MDMX expression levels were detected between various CLL categories, obtained according to IGHV gene status, FISH groups, and CD38, CD49d or ZAP-70 expression. *Conclusions.* in keeping with an over-expression of MDM4/MDMX detected in primary samples from several solid tumors, including glioblastoma, retinoblastoma, breast, colon, and lung cancers and preclinical studies indicating the anti-neoplastic effects of MDM4/MDMX down-regulation in murine lymphoma models, MDM4/MDMX turned out to be universally over-expressed by CLL cells compared to normal B cells, despite its peculiar over-expression in a subset of Nutlin-3 "non-responder"p53^{wt} CLL. This observation contributes to identify MDM4/MDMX as a potentially useful new therapeutic tar*get* also for CLL.

0743

PROLIFERATIVE CELLULAR COMPARTMENT IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL): CHARACTERIZATION AND PROGNOSTIC SIGNIFICANCE

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Background. Historically chronic lymphocytic leukemia (CLL) has been considered a non-proliferative disease characterized by the accumulation of resting mature B-lymphocytes. However, recent findings are questioning this concept, suggesting the existence of a significant cell proliferation. Furthermore, CLL cells present in bone marrow (BM) and lymph nodes (LN) seem to be more prone to proliferation than those present in the peripheral blood (PB). The amount of cell proliferation and its prognostic significance has not been properly analyzed. *Aims.* To determine, both in BM and PB, the expression of genes involved in proliferation, to phenotypically characterize the leukemic subpopulation that proliferates and to analyze the relationship between the degree of proliferation and clinical characteristics of the disease. *Methods.* Gene expression profiling of proliferation genes and the amount of cell proliferation in different tissue compartments (BM and PB) were examined in patients with CLL. In isolated CD19/CD5^{+} tumoral cells from 17 paired PB and BM samples, expression of genes (n=93) involved in the initiation and development of the cell cycle was analyzed by low-density TaqMan® arrays. The amount of proliferative (Ki67 positive) CLL cells was measured by flow cytometry in 50 paired samples. In addition, coexpression of molecules associated with cellular activation (CD38, CD71, CD69), adhesion (CD49d), chemokine receptors (CXCR4, CXCR3), interaction between T and B cells (CD86), signaling (ZAP-70), and Toll-like receptors (TLR9) was compared between Ki67^{+} and Ki67^{-} CLL subpopulations. Finally, the degree of proliferation was correlated with the main clinical and biological characteristics.

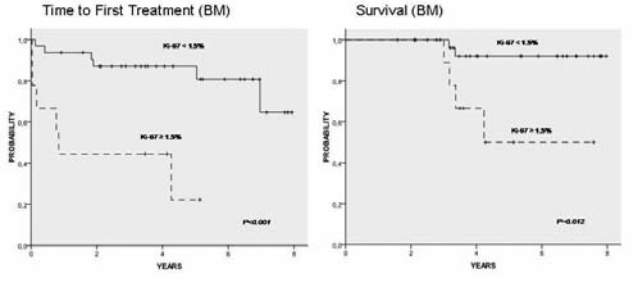

Figure 1. Shorter time to first treatment and inferior survival in patients with increased Ki67(+) CLL cells.

Results. As assessed by gene expression profiling, genes involved in the initiation and progression through the cell cycle had higher expression in CLL cells from BM than in those from PB. Moreover, the percentage of proliferating (Ki-67^{+}) CLL cells analyzed by flow cytometry was significantly higher in BM than in PB. The Ki-67^{+} CLL cells had different immunophenotypic characteristics (increased expression of ZAP-70, CD38, CD49d, CD5, CD86, CD71, and TLR9 and decreased expression of CXCR4 from the non-proliferating (Ki-67^{-}) CLL cells. Adverse clinical and biological prognostic markers (short lymphocyte doubling time, lymph node areas involved, lymphocytosis, and increased expression of ZAP-70, CD38 and CD49d) were associated with the degree of proliferation, and also, the amount of Ki-67^{+} CLL cells correlated with a shorter time to first treatment and a worse survival (Figure). *Conclusions.* Altogether, these results challenge, once more, the concept of CLL as an accumulative disease rather than a proliferating one by showing that a significant fraction of the CLL clone has a proliferating phenotype and, moreover, that a higher degree of proliferation correlates with a more aggressive disease.

0744

IN CHRONIC LYMPHOCYTIC LEUKEMIA THE AMOUNT OF PROLIFERATION CENTERS IN TISSUE-BIOPSIES CORRELATES WITH UNFAVORABLE CYTOGENETIC ABNORMALITIES

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Background. In chronic lymphocytic leukemia (CLL) molecular cytogenetic abnormalities may identify specific disease entities and may be associated with distinct prognosis. Currently, peripheral blood is the preferred source to assess the biological risk. However, fluorescence in-situ hybridization (FISH) on paraffin-embedded fixed tissues (PEFT) may also be used to study cytogenetic abnormalities in CLL lymph-node where the "proliferation center" (PC) is the histopatologic hallmark. PCs probably represent the sites where T-dependent immune responses to unknown antigens promote clonal B cell selection and expansion. *Aims.* i) to analyze the sensitivity and reproducibility of FISH on PEFT, ii) to correlate specific chromosome lesions to PC extension; iii) to estimate the frequency of chromosome lesions on lymph node samples. Method: 183 CLL cases (including 20 Richter syndrome, RS) arranged in 5 tissue macro-arrays (TMAs) were analyzed by FISH for deletions involving 11q23/ATM, 13q14 and 17p13/p53; trisomy 12; and translocations involving the immunoglobulin gene (IgH) on band 14q32. Cytogenetic features were correlated to 2 histologic patterns: 1) PC-rich group including CLL cases with confluent and large PCs and RS cases and 2) typical group with scattered, well-distinct PCs. *Results.* Assessable data with the complete 5 probe-panel were obtained in 101/183 cases (55,1%); in 58 cases the results were partial and in 24 cases no data were collected for all the probes mainly because of insufficient numbers of cells on TMA samples. Chromosomal aberrations were detected in 79/101 cases (78.2%). The most frequent abnormality was 13q- (36.7% of the cases), followed by 14q32 translocations (30.8%), 11q- (24.7%), +12 (19.5%) and 17p- (15.6%). Ten cases showed 14q32/IgH gene extra-copies (3-5 signals in 10-30% of the nuclei). 17p-, +12 and 14q32/IgH translocations were more frequently encountered in the PCs-rich group (P<.001, .030 and .043 respectively). The PC-rich group was significantly associated to "high-risk"cytogenetic abnormalities (11q- and/or 17p-; P=.001) and to a higher number of abnormalities (≥ 2; P=.001). The difference persists even if RS cases were excluded. *Conclusions.* FISH on TMAs is a reproducible and a feasible tool for the evaluation of cytogenetic abnormalities in CLL allowing for a decreased both time consumption and experimental variability. The incidence of cytogenetic abnormalities in our series resembles that of historical series with exception of 14q translocation and 17p- possibly due to a higher number of patients with resistant or progressive disease. The higher occurrence of unfavourable cytogenetic features in PC-rich lymph nodes suggest that PCs may be sites where, due to an antigenic stim-

uli, B cells are induced to proliferate thus conferring genetic instability. Further studies will clarify if cytogenetic abnormalities occurring in lymph-node and bone marrow PCs precede their appearance in the peripheral blood thus supporting the idea that at tissue level PC is crucial for triggering clonal proliferation nourishing the accumulation compartment.

0745

THE IMPACT OF THE MICROENVIRONMENT ON CLL CELLS

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Background. Chronic lymphocytic leukaemia (CLL) is characterized by high numbers of CD5/CD19 positive cells in the peripheral blood (PB). These cells show prolonged survival and accumulate in the PB and lymphoid organs of the patients. Both, intrinsic defects in the regulation of programmed cell death (apoptosis) as well as an altered, survival-stimulating microenvironment are discussed as major pathogenic factors of CLL. This is emphasised by the fact that CLL cells rapidly undergo apoptosis *in vitro*, but cell survival can be maintained by stromal cell co-cultures for months. *Aims.* Our studies aimed at the molecular characterization of microenvironmental factors that contribute to the abnormal long survival of CLL cells to identify potential targets for novel therapy strategies for CLL patients. *Methods.* To elucidate the impact of microenvironmental factors on the survival of CLL cells, we performed expression profiling of peripheral blood mononuclear cells (PBMC) from CLL patients in three survival inducing culture conditions: a) co-culture with the human bone marrow-derived stromal cell line HS-5, b) culture in conditioned medium of HS-5 cells, and c) high cell density cultures of CLL cells. Additionally, we quantified protein levels of 174 different cytokines in the supernatant of CLL/HS-5 co-cultures by performing antibody arrays. Data validation was performed by qRT-PCR and ELISA. The impact of the candidate gene CCL2 on cell survival, apoptosis and migration was analyzed by flow cytometry after annexin V/7-AAD staining and in transwell assays. *Results.* Expression profiling revealed that there is a large overlap of differentially regulated genes in the three survival inducing culture conditions tested. Among these, we identified many cytokines which were upregulated on transcriptional level. These data could be validated on protein level by cytokine array analysis. Interpretation of the array data revealed CCL2 as one potential candidate gene. By qRT-PCR and ELISA with CD19-sorted B-cells, CD14-sorted monocytes and unsorted PBMC we could observe an upregulation of CCL2 expression and secretion by monocytes in the co-cultures. To evaluate the importance of this chemokine in CLL *in vivo* we have quantified CCL2 serum levels in the PB of patients and healthy donors and detected significantly elevated levels in CLL patients. We could further show by ELISA that CLL cells are able to induce CCL2 production in monocytes. Using transwell assays with recombinant CCL2, conditioned medium of CLL/HS-5 co-cultures, or serum of CLL patients as chemotactic stimuli, we observed induced migration of monocytes, which could be reduced by adding neutralizing antibodies against CCL2. *Conclusions.* Accessory cells within the CLL microenvironment play a critical role for the survival of CLL cells by releasing soluble factors. CLL cells are able to actively contribute to a favourable microenvironment by inducing expression of CCL2 in monocytes. This causes attraction of additional monocytes, which release a variety of different cytokines that contribute to the better survival of the CLL cells. Therefore, neutralization of monocytes-derived factors like CCL2 should be evaluated as a novel approach to improve treatment of CLL patients.

0746

EXPRESSION OF MIR-15A AND MIR-16-1 IN PRIMARY CLL

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Background. Heterozygous or homozygous loss of a minimally deleted region (MDR) of 13q14 spanning miR-15a and miR-16-1 is the most common genomic abnormality in CLL, occurring in approximately 70% of cases. Growing evidence supports a role for deregulation of miR-15a and miR-16-1 and hence of their downstream targets as a mechanism of action for a selective advantage conferred by 13q14 deletion. Expression of a DLEU2 transcript variant has been suggested as a mechanism of miR-15a and miR-16-1 production and variable methylation of the DLEU2 promoter has been reported in CLL. *Aims.* To investigate the relationship between expression levels of DLEU2, miR-15a and miR-16-1 and methylation of the CpG islands within the 13q14 MDR in primary CLL samples. *Methods.* To reduce effects of intra-clonal variation, samples from 61 CLL cases were selected based on 13q14 deletion status of either no loss (n=25), >80% homozygous loss (n=27) or >80% heterozygous loss (n=9) by FISH. Relative quantification of DLEU2, miR-15a and miR-16-1 expression levels was performed by taqman RT-PCR, together with quantification of DNA methylation of five CpG islands associated with13q14 MDR genes by bisulphite pyrosequencing. Results. Expression of DLEU2, miR-15a and miR-16-1 was 3-7 fold lower in homozygous loss samples compared to both heterozygous and no loss samples ($P<0.01$). Samples with one or more intact copies of the MDR displayed expression values across a >6-fold range for DLEU2 and >30-fold range for miR-15a and miR-16-1, however no significant difference between heterozygous loss and no loss samples was detected. In the no loss samples miR-15a and miR-16-1 expression levels showed a positive correlation with each other ($P<0.001$) and with DLEU2 expression ($P=0.002$). In the heterozygous loss samples, the same positive correlation was seen between miR-15a and miR-16-1 expression ($P<0.001$) but neither miR-15a nor miR-16-1 correlated with DLEU2 expression ($P>0.9$). Four of five CpG islands analysed showed hypomethylation in all 61 cases. Variable methylation 0-98%, mean 58.8±31.6% was detected within the CpG island associated with the transcription start site for DLEU2. Methylation of this island was not significantly different between deletion groups and did not correlate with expression levels of DLEU2, miR-15a or miR-16-1 transcripts. *Summary/Conclusions.* Considering the samples with high levels of homozygous loss as having almost zero expression of the three transcripts, those with one or more intact copies of the MDR clearly display expression of all three genes. This fact, together with the range of expression levels in the heterozygous loss samples, confirms that loss of one allele does not silence expression of miR-15a and miR-16-1. The close correlation between miR-15a and miR-16-1 transcript levels is suggestive of a linked mechanism for their regulation and it is of note that this may be related to DLEU2 expression in cells with two copies of the MDR but not in those with heterozygous loss. The confirmation of variable methylation of the DLEU2 promoter in the absence of an obvious association with expression suggests that further investigation of the DNA methylation in this region is warranted.

0747

COMPLEMENT-DEPENDENT LYSIS OF MAB-OPSONIZED B CELLS CAN INCREASE RESISTANCE OF NON-OPSONIZED B CELL LINES AND PRIMARY B CELLS TO SUBSEQUENT ATTACK

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Background. Complement (C)-dependent cytotoxicity (CDC) is a major effector mechanism utilized by rituximab (RTX) and ofatumumab (OFA) in cancer immunotherapy. We recently reported that during mAb-mediated CDC, B cell lines, and B cells from chronic lymphocytic leukemia (CLL) patients, release long, thin streamers (aka tunneling nanotubules) from their surfaces. *Aims.* We now report that streamer generation requires calcium entry into cells, suggesting that signaling mediated by streamers may promote intercellular communication. Therefore we investigated whether CDC of mAb-opsonized B cells (sacrificial cells), and the accompanying streaming reaction can induce resistance to CDC in neighboring non-opsonized B cells (indicator cells) that are later subjected to mAb opsonization and C attack. *Methods.* PKH-dyed indicator cells were combined with non-opsonized or mAb-opsonized sacrificial cells and reacted with normal human serum (NHS) for 4 hrs. Additional NHS and mAb were then added, and CDC of the indicator cells was determined. This paradigm allows us to investigate whether the first "wave"of C attack on opsonized sacrificial cells induces resistance to the second wave of C and mAb attack on the indicator cells. *Results.* CDC of indicator Z138 cells was 91% if non-opsonized sacrificial Z138 cells were present in the 4 hr incubation, but CDC was only 63% if OFA-opsonized sacrificial Z138 cells were present, demonstrating development of resistance to CDC in indicator cells. OFA was more effective than RTX at overcoming CDC resistance of indicator cells. Challenge of indicator Daudi cells with RTX gave 85% or 50% CDC using naive or OFA-opsonized sacrificial Daudi cells, respectively, whereas CDC after challenge with OFA was 90% and 85%, respectively. Malignant B cells from CLL patients also acquire

substantial CDC resistance in this system. Challenge of indicator CLL B cells with OFA gave 63% or 21% CDC with non-opsonized or OFA-opsonized sacrificial Daudi cells, respectively. In order to examine the effects of calcium in these reactions, we substituted NHS-Mg-EGTA for NHS in the paradigm. Under these conditions (calcium is chelated by EGTA) the opsonized sacrificial cells are killed by the alternative pathway of C, but the cells do not release streamers. Moreover, indicator cells reacted with OFA-opsonized sacrificial cells in NHS-Mg-EGTA did not acquire resistance to classical or alternative pathway CDC, strongly suggesting that streaming and calcium influx into sacrificial and/or indicator cells play key roles in development of resistance to CDC. *Summary/Conclusion.* Exposure to sacrificial cells undergoing CDC protects indicator cells (B cell lines and primary CLL cells) from subsequent CDC upon challenge with OFA or RTX and C. Loss of protection when sacrificial cells are killed by the alternative pathway of C suggests that calcium fluxes play a key role in the development of resistance to CDC.

0748

THE VEGF-STAT3 AXIS IS ESSENTIALLY INVOLVED IN THE BONE MARROW STROMAL CELL-MEDIATED CLL CELL SURVIVAL SUPPORT AND QUALIFIES AS A TARGET FOR SELECTIVE CLL THERAPY

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Background. The major pathophysiological feature of chronic lymphocytic leukemia (CLL) cells is there resistance towards apoptosis. Thereby, the bone marrow (BM) microenvironment has a substantial role as CLL cells die rapidly when taken out of their natural habitat and are placed in culture. The vascular endothelial growth factor (VEGF) has frequently been associated with the resistance of CLL cells towards apoptosis. Several bystander cells in the BM of the patient such as stromal cells are capable of producing VEGF in high amounts. *Aims.* To describe the effect and functional background of VEGF in the interplay between CLL cells and the BM stromal cell line HS5 to potentially discover targeted for a selective CLL therapy. *Methods.* Primary cells were isolated from peripheral blood of CLL patients and healthy volunteers. Real time PCR was used for mRNA analysis and immunoblotting for protein detection. Secreted VEGF was measured by ELISA. Phosphorylation of the VEGF-receptor 2 (VEGF-R2) was analysed using intracellular phospho flow cytometry. VEGF-specific siRNAs were used to knock down VEGF in HS5 cells. VEGF-R- (GW 786034) and STAT3-inhibitor (S23I-201) were added to HS5/CLL coculture in several concentrations. Cell survival was assessed by annexin V/PI negativity. *Results.* CLL cells express and secrete VEGF in significantly higher levels as healthy B-cells. They also possess constitutively phophorylated VEGF-R2. When kept in monoculture this phosphorylation is lost over time, simultaneously CLL cells die. Vice versa, cocultivation with HS5 cells restores the phosphorylated VEGF-R2 and maintains CLL cell survival. When VEGF is blocked in this coculture setting by either a VEGF-neutralizing antibody (external blockage), by the VEGF-R inhibitor GW786034 or by siRNA-mediated VEGF downregulation in HS5 cells (internal blockage) , the survival advantage is significantly reduced. As a potential mechanism of VEGF-induced CLL cell survival support, STAT3 activation via phosphorylation on tyr705 could be demonstrated. This phosphorylation was induced by exogenous rhVEGF stimulation and also by coculture with HS5. In both cases, VEGF-R blockage by GW 786034 treatment reversed tyr705 phosphorylation and the increased expression of STAT3 targets, such as cyclinD1 and Bcl_{XL}. With this background also the potential of STAT3-inhition to induce CLL cell apoptosis was assessed. Both, the VEGF-R inhibitor GW786034 and the STAT3 inhibitor S3I-201 were capable of significantly reducing the HS5-mediated survival advantage. *Conclusions.* The VEGF-signaling pathway seems to be essentially involved in the survival of CLL cells within their natural microenvironment, as its external (neutralizing antibody) and internal (siRNA-mediated VEGF knock down in HS5 cells) inhibition significantly reduced the HS5-mediated survival advantage. STAT3 activation could be identified as a potential downstream effector. The small molecule VEGF-R inhibitor GW 786034 and the STAT3 inhibitor S3I-201 showed promising effects in inducing cell death of CLL cells within the survival-supporting atmosphere of HS5 coculture, suggesting the VEGF-STAT3 signaling axis to be of high interest for a targeted CLL therapy with them aim of overcoming the apoptotic block.

0749

ANGIOPOIETIN-2 PLASMA DOSAGE PREDICTS TIME TO FIRST TREATMENT (TTFT) AND OVERALL SURVIVAL (OS) IN CHRONIC LYMPHOCYTIC LEUKEMIA

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The clinical relevance of Angiopoietin-2 (Ang2) in CLL was previously suggested by the association between high Ang2, more advanced disease and shorter progression-free survival reported in small series of patients. Here, we further investigate the prognostic role of plasma level of Ang2 in a multicentric study with a large cohort of CLL patients. We measured Ang2 glycoprotein levels in plasma samples from 316 CLL patients using an ELISA method and we investigated its prognostic role in relation to time to first treatment (TTFT) and overall survival (OS). Ang2 dosage ranged from 972 to 17281 pg/mL (median, 2061 pg/mL). The best cut-off point, generated by ROC analysis and Youden's index (state variable, treating), was 2459 pg/mL and divided our cohort in two subsets (high Ang2 and low Ang2) composed by 100 (31.6%) and 216 (68.4%) patients, respectively. The median TTFT resulted significantly shorter (P<.001) in the high Ang2 subgroup (77.5 months) than in the low one (179.2 months). Cox univariate analysis identified Ang2 ≥2459 pg/mL as a predictor of reduced TTFT (HR 2.437; 95%CI 1.621-3.664, P<.001) as well as advanced Binet stage, unmutated IGHV status, high CD38, ZAP-70 and CD49d expression, intermediate/high cytogenetic risk and high β2 microglobulin (P<.001 in all instances). Multivariate analysis confirmed that high Ang2 levels were an independent prognosticator for TTFT (HR 1.739; 95%CI 1.059 - 2.857; P=.029) together with inter/high FISH risk (P<.001) and unmutated IGHV status (P=.002). Comparing OS between high Ang2 and low Ang2 subgroup, we found that 26% of high Ang2 patients were dead at 10-years from diagnosis, in contrast with 7% of low Ang2 ones (P=.002). In univariate analysis, Ang2 ≥ 2459 pg/mL resulted to be a predictor of poor OS (HR 3.566; 95%CI 1.496 - 8.499; P=.004) as well as most of the other known unfavorable prognosticators. Significant association was found between high Ang2 plasma level and Binet stages B-C (P<.001), high β2 microglobulin (P<.001), unmutated IGHV status (P<.001), high CD38 and ZAP-70 expression (P<.001 and P=.003, respectively) and inter/high cytogenetic risk (P=.005). However, a relevant percentage of patients showed high Ang2 levels in the presence of favorable markers and vice versa: in this cases, defined as discordant, Ang2 helps to refine prognosis identifying CLL subgroup with precocious need for treatment and reduced survival despite characterized by favorable prognostic factors. Finally, we studied two serial plasma samples collected from 36 patients (median interval, 19 months; range, 3-30 months) and we did not find significant changes in Ang2 level between the two measurements, both for the 7 patients treated inside this interval (P=.612) and for the 29 cases untreated (P=.347). These data first demonstrate the prognostic role of Ang2 plasma level for TTFT and OS in CLL, and shows how Ang2 contributes to refine the prognostic assessment of CLL and also helped to refine prognosis among CLL subsets with both high and low VEGF plasma levels. Ang2 plasma level may be a useful independent prognosticator for CLL.

0750

XIAP INHIBITION AS A NEW PARADIGM TO INDUCE APOPTOSIS IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Evasion of apoptosis is a hallmark of chronic lymphocytic leukemia (CLL), calling for new strategies to overcome apoptosis resistance. We therefore investigated whether targeting the antiapoptotic protein XIAP

by small molecule inhibitors sensitizes CLL cells for apoptosis. Here, we provide first evidence that XIAP inhibitors in combination with the death receptor ligand TRAIL present a new approach to synergistically trigger apoptosis in CLL even in subgroups with resistant disease. Analysis of apoptosis regulatory proteins reveals that XIAP, cIAP1 and cIAP2 are expressed at high levels in primary CLL samples. Proofs of concept studies in CLL cell lines demonstrate that subtoxic concentrations of several distinct XIAP inhibitors significantly enhance TRAIL-induced apoptosis. By comparison, no sensitization for death receptor-induced apoptosis is observed in the presence of a structurally related control compound that only weakly binds to XIAP, demonstrating the specificity of the sensitization effect of XIAP inhibitors. Importantly also in primary CLL samples, subtoxic concentrations of XIAP inhibitors act in concert with TRAIL to trigger apoptosis in 18 of 27 cases (67%), whereas primary CLL cells are resistant to treatment with TRAIL alone. Analysis of combination index reveals that this interaction of XIAP inhibitor and TRAIL is highly synergistic. Mechanistic studies in primary CLL cells show that the addition of XIAP inhibitor profoundly enhances TRAIL-induced cleavage of caspase-3 into active fragments and significantly increases caspase-3 enzymatic activity. The broad range caspase inhibitor zVAD.fmk completely blocks apoptosis in response to combination treatment with XIAP inhibitor and TRAIL, pointing to caspase-dependent apoptosis. Intriguingly, the cooperative interaction of XIAP inhibitor and TRAIL is even evident in several distinct subgroups of CLL patients with poor prognostic features, including patients with 17p deletion, TP53 mutation, chemotherapy-refractory disease or unmutated VH genes. Interestingly, we found that cases with unmutated VH genes are significantly more sensitive to XIAP inhibitor- and TRAIL-induced apoptosis compared to VH gene mutated samples, pointing to a role of B-cell receptor signaling in the regulation of apoptosis in CLL cells. In conclusion, we provide first evidence that XIAP inhibitors in combination with TRAIL present a novel strategy to trigger apoptosis even in resistant forms and poor prognostic subgroups of CLL. These findings have important implications for the development of innovative approaches to overcome the intrinsic resistance to apoptosis in CLL. Since IAP inhibitors as well as TRAIL receptor agonists as single agents are currently under evaluation in early clinical trials, it is feasible that such combination protocols of XIAP inhibitors and TRAIL could be translated into clinical application in CLL.

0751

GLIOTOXIN INDUCES APOPTOSIS IN CLL CELLS BY A MECHANISM INVOLVING INHIBITION OF NOTCH2 ACTIVITY

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Background. NOTCH2 belongs to a highly conserved family of transmembrane receptors that regulate a wide variety of cellular differentiation processes including stem cell renewal, binary cell fate decisions, and adult tissue homeostasis. We have recently shown that constitutively activated NOTCH2 is involved in the high expression and aberrant regulation of CD23 in CLL cells and there is emerging evidence that NOTCH2 has also an antiapoptotic role in the leukemic lymphocytes. In murine B-cell development, constitutively activated Notch2 leads to the selective development of $Cd5^+$ (B-1a) B-lymphocytes whose human equivalents are considered as one of the possible progenitors of CLL cells. Therefore, targeting NOTCH2 might be of therapeutic relevance in CLL. *Aims.* The aim of this study was to identify compounds that interfere with NOTCH2 signaling at the transcription factor level. Several compounds have been tested and we focused the investigations on the secondary fungal metabolite Gliotoxin which has been reported to affect the viability of splenocytes. *Methods.* The nuclear NOTCH2 activity in CLL samples was determined by electrophoretic mobility shift assays (EMSA). FACS analysis was used to quantify the surface expression of the NOTCH2 target gene CD23 and to measure the percentage of apoptotic cells. *Results.* Gliotoxin (0.2μM) completely blocked the activity of nuclear NOTCH2 in CLL cells irrespective of their prognostic marker profile. The inhibition of NOTCH2 signaling by Gliotoxin was associated with downregulation of surface CD23 expression (mean±SD: 42±33 *vs.* 86±14; n=20) and induction of apoptosis (mean±SD: 70±29 *vs.* 13±14). In contrast, normal PBMCs and nuclear NOTCH negative leukemic cell lines (Jurkat, KL562, SH3, RL7) were less sensitive to Gliotoxin. To test whether Gliotoxin directly interferes with the assembly of the NOTCH2 transcription factor complex, we added Gliotoxin to the EMSA reaction and found that Gliotoxin inhibits the formation of DNA-bound NOTCH2 complexes in a dose dependent manner. *Summary/Conclusions.* We identified Gliotoxin as potential NOTCH2 transactivation inhibitor and showed that Gliotoxin possesses a strong antileukemic activity in CLL cells.

0752

THE PARA-ISOMER OF NITRIC OXIDE-DONATING ACETYL SALICYLIC ACID (P-NO-ASA) INDUCES APOPTOSIS IN CLL CELLS IN VITRO AND SIGNIFICANTLY REDUCES TUMOR GROTH IN A CLL-LIKE XENOGRAFT MOUSE MODEL

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Background. Chronic lymphocytic leukemia (CLL) is characterized by an accumulation of mature, non functional B cells. WNT/β-catenin/Lef-1 signalling appears to be constitutively and aberrantly activated in these cells. Furthermore, several compounds related to the non-steroidal anti-inflammatory drugs (NSAIDs) are reported to destabilize β-catenin, therefore inducing apoptosis in β.catenin/LEF-1 active cancers in-vitro. Clinical studies with such substances generated disappointing results as therapeutically effective plasma concentrations could not be reached without producing significant toxicities. Recently, nitric oxide-donating acetyl salicylic acid (NO-ASA) has been developed, which achieves high plasma levels in doses not leading to any relevant side effects in humans. *Aim.* To test two positional isomers of NO-ASA for their ability to selectively induce apoptosis in primary CLL cells *in vitro* and for their *in vivo* efficacy in a CLL-like xenograft mouse model. *Methods.* Primary CLL cells as well as healthy B-cells were treated with varying concentrations of para-(p) and meta-(m) NO-ASA. Cytotoxicity was assessed by microscopic cell viability testing and measurement of the ATP content. Induction of apoptosis was investigated by Annexin V-FITC/Propidiumiodid (PI) staining and immunoblotting of caspases 9, 3 and PARP. Further, protein amount of β-catenin and its specific target gens like cyclin D1, C-MYC and LEF-1 was evaluated by immunoblot analysis. To evaluate the in-vivo efficacy, both substances were tested in a CLL like xenograft mouse model. *Results.* The para-isomer showed a selective cytotoxic effect, whereas the meta-isomer of NO-ASA did not alter survival in CLL cells. At 10 μM for 24 hours p-NO-ASA reduced survival of CLL cells to 46.3±10.1%. Time course experiments revealed that this effect was almost completely achieved as early as 6 hours after treatment (53.4±16.6% survival). Immunoblot analysis showed that p-NO-ASA activates caspase 9, 3 and cleaves PARP, at concentrations of around 10 μM. Furthermore, β-catenin was cleaved and its target genes cyclin D1, C-MYC and LEF-1 were simultaneously downregulated. Pre-treatment with a caspase-inhibitor prevented both, β-catenin cleavage and reduction of protein levels of its target genes. In the xenograft CLL-like mouse model exclusively p-NO-ASA featured a strong antitumor efficacy with an IR_{max} value of 83.1%. As early as 9 days after p-NO-ASA treatment the tumor volume was significantly reduced compared to vehicle control. The meta-isomer showed no significant anti-neoplastic effect. No gross side effects were observed. *Summary/Conclusions.* Our findings show that exclusively the para-isomere of NO-ASA selectively induces Caspase-mediated apoptosis in CLL cells. The mechanism of action might include inhibition of β-catenin/LEF-1 signaling by β-catenin cleavage, since we observed a downregulation of specific target genes at the protein level. In addition, p-NO-ASA resulted in strong inhibition of tumor growth in a xenograft CLL-like mouse model after oral administration, while being well tolerated. Hence, p-NO-ASA might be a valuable compound for the treatment of CLL. Further investigations to determine the exact mechanism of action and the specific difference between the positional isomers are of high interest.

0753

ACADESINE INDUCES DOSE-DEPENDENT CITOTOXICITY IN CHRONIC LYMPHOCYTIC LEUKEMIA REGARDLESS OF IMMUNOGLOBULIN MUTATIONAL STATUS AND ZAP70 EXPRESSION

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Background. The precursor of nucleotide biosynthesis acadesine or 5-aminoimidazole-4-carboxamide (AICA) riboside induces apoptosis in chronic lymphocytic leukemia (CLL) and other lymphoproliferative dis-

eases such as splenic marginal zone lymphoma and mantle cell lymphoma. This effect is selective for B-CLL cells, at least *ex vivo*, however, there is no evidence of whether this cytotoxic effect is associated with prognostic variables such as ZAP-70 expression or IgVH mutational status. *Aim.* To analyse *ex vivo* the cytotoxic effect of acadesine on peripheral blood CLL cells and non CLL cells and correlate it with variables of prognostic value such as: cytogenetic alterations, ZAP-70 expression and IgVH mutational status. *Methods.* Cryopreserved cells from 22 patients diagnosed with CLL were incubated *ex vivo* with acadesine at 0.2, 0.5, and 1mM for 24 hours. Viability was determined by flow cytometry using Annexin V-FITC and DAPI staining combined with CD19-PE and CD3-PerCP to differentiate cell viability of B- and T-cells. Cells were considered sensitive to the drug when the percentage of acadesine-induced apoptosis was equal to or higher than 15% with respect to the viability of control cells. The mutational status of IgVH genes was determined by RT-PCR amplification using a set of six VH family-specific primers (VH1 through VH6) along with primers complementary to the constant region (IgM and IgG). Products were directly sequenced from both strands using the Big Dye Terminator Cycle Sequencing Ready Reaction (version 3.1, Applied Biosystems). Sequencing analysis and alignments were performed with use of V-QUEST software and the online international immunogenetics information (IMGT) data library. Samples in which fewer than 2 percent of base pairs differed from those of the consensus sequence have been considered unmutated. ZAP-70 expression was quantified by flow cytometry (cut-off: 20%) and cytogenetic alterations associated with CLL (trisomy 12, del13q, del17p and del11q) were determined by FISH. Results. After 24h of *ex vivo* incubation, 0.2 mM acadesine induced a significant cytotoxic effect (>15%) in 19 out of 22 patients (86%). Higher concentrations, 0.5mM and 1mM, induced a significant effect in 95% (21 of 22 patients) and 100% (22 of 22 patients), respectively. The viabilities (mean±SD) of the different culture conditions are shown in the Table 1. The cytotoxic effect induced by acadesine was correlated with the IgVH mutational status and with ZAP-70 expression. No significant differences were observed between unmutated (n=10) and mutated (n=12) cases or between ZAP-70 positive (n=8) and ZAP-70 negative cases (n=14). In addition, no significant differences were observed between cases showing no cytogenetic alterations (n=7) and cases displaying del 13q (n=10). Interestingly, 2 cases showing deletion of 17p were sensitive to treatment with acadesine, in agreement with previously published studies showing that acadesine-induced apoptosis is independent of p53. Conclusions. Acadesine induces apoptosis in B-cells from CLL regardless of ZAP-70 expression, IgVH mutational status and presence or absence of cytogenetic alterations.

Table 1.

	% Viability	%acadesine-induced apoptosis
Control cells	68.58 ± 15.31	-
0.2 mM acadesine	59.12 ± 19.01	14.20 ± 17.50
0.5 mM acadesine	34.79 ± 21.25	50.86 ± 24.30
1 mM acadesine	20.23 ± 19.29	70.21 ± 29.51

0754

INHIBITION OF DNA-PK SENSITIZES A PROPORTION OF CLL CULTURES TO FLUDARABINE, REGARDLESS OF THE TP53/ATM STATUS

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Background. Defects in cooperating tumor-suppressor genes TP53 or ATM significantly deteriorate treatment options for CLL patients. DNA-dependent protein kinase (DNA-PK) is responsible for the process of nonhomologous end-joining DNA repair, through which some cancer cells can escape a chemotherapy-induced apoptosis. Chemical inhibition of DNA-PK was shown to sensitize resistant CLL cells to chemotherapeutics, including some cultures with del(17p) (TP53 locus) or del(11q) (ATM locus). Another (non-CLL) study have shown, however, that inhibition of DNA-PK does make sense only in the ATM-deficient setting in cancer cells, when DNA repair is completely blocked (Jiang *et al.,* Genes Develop 2009; 23: 1895-1909). *Aims.* To assess an effect of DNA-PK inhibition in the high-risk CLL cultures treated *in vitro* with a nucleoside analog fludarabine. *Methods.* PBMNC isolated from CLL patients were exclusively used. The fraction of CLL lymphocytes comprised at least 85% and viability before treatment was >80% in all samples. The cells were treated for 1h with or without the synthetic DNA-PK inhibitor NU7026 (10 μM) and subsequently exposed to four different concentrations of fludarabine (25; 6.25; 1.6 and 0.4 μg/mL) for 48h. A metabolic WST-1 assay was used to assess a cell viability. Two-way analysis of variance (ANOVA) was used to determine a significance of the NU7026 pre-treatment; viability after treatment with NU7026 on its own was set up at 100% (i.e. the same as fully non-treated control). Quantitative real-time PCR (qRT-PCR) was used to monitor an induction of pro-apoptotic (PUMA, BAX), cell-cycle regulatory (p21) and DNA-damage repair (GADD45) genes. *Results.* Forty-four CLL cultures were tested; 15 with p53 inactivation (deletion by FISH and/or mutation by FASAY coupled to sequencing), 15 with heterozygous ATM deletion (by FISH) and 14 without any high-risk abnormality (wt). DNA-PK inhibitor on its own reduced a viability in most of the cultures, with the lowest effect in TP53-affected cells (viability decreased on average to 73%), followed by ATM-deleted (63%) and wt (61%) samples. A sigificant (P<0.01) sensitization effect of DNA-PK inhibitor to fludarabine was observed in 40% (6/15) of p53-defected samples, 20% (3/15) of ATM-deleted samples and 14% (2/14) of wt cultures. The sensitized samples were affected much less with the NU7026 on its own than the rest of cultures (everage viability 88% *vs.* 59%, respectively) and were substantially more resistant to fludarabine at the concentration resembling a clinical situation, i.e. 1.6 μg/mL (everage viability 78% *vs.* 53%, respectively). qRT-PCR was performed in 11 cultures. All tested genes were readily induced by fludarabine and fludarabine with NU7026 (to the very same level in particular sample) in all wt cultures (n=3), all ATM-deleted cultures (n=6) and in sample with sole TP53 mutation (n=1); sample with complete TP53 inactivation was refractory to any induction. Summary/*Conclusions.* Our results support the view that sensitization of CLL cells using DNA-PK inhibition is feasible and may be expected in a case when the cells are resistant to fludarabine. On the contrary, the sensitization may not be dependent in principle on particular genomic abberations.

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0755

THE ROLE OF VIMENTIN IN CHRONIC LYMPHOCYTIC LEUKAEMIA

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Background. Chronic Lymphocytic Leukaemia (CLL) is a heterogeneous disease and can be characterized by low proliferation rates and defective apoptotic pathways. Within CLL, two subtypes have been defined by the degree of somatic hypermutation, unmutated (UM) or mutated (M) in the variable region of the immunoglobulin heavy chain (IGHV) gene. Recent data has shown that expression of Wnt genes, associated receptors and downstream signalling molecules are deregulated in CLL compared to normal B cells. The Wnt family of growth factors are secreted glycoproteins, involved in the proliferation and differentiation of normal and malignant cells. Wnts can activate various pathways such as the non-canonical Wnt/$Ca2^+$ pathway, which can lead to the upregulation of Vimentin expression. Vimentin is a type III dominant cytoskeletal protein and intermediate filament, important for determining rigidity of lymphocytes, and is thought to be defective in lymphocytes from CLL patients, potentially contributing to smear cell formation. Recent reports suggest Wnt signalling upregulates Vimentin and that Vimentin expression is higher in leukaemic cells from patients with aggressive disease. *Aims.* This study aims to investigate the role Vimentin plays in CLL. *Methods.* $CD19^+$ cells were purified from peripheral blood (PB) of CLL patients (Miltenyi-autoMAX), IGHV and cytogenetic status was determined. RNA (Trizol), protein lysates and cytospins were prepared. Expression of Vimentin, Wnt genes and associated Wnt signalling pathway genes in CLL cell lines (Mutated-E95, B4, PGA1 HG3, EHEB, Unmutated-HG3, Wac3, CII) and patient samples

was assessed using RQ-PCR and Western Blotting. Co-IP of Vimentin (Millipore) and pERK (Santa-Cruz) from protein lysates was performed. Vimentin distribution and expression in cell lines and patient PB samples and primary lymphoid tissue was visualized by IHC, fluorescent staining (Alexa-Fluor) and Confocal Microscopy. *Results.* Gene expression profiling identified a signature of upregulated Wnts, Wnts 4, 5a, 16b, receptors Fzd3, ROR1, RYK and regulators sFRPs 1,2 and 4, in our cell lines and patient cohort, indicating non-canonical Wnt/$Ca2^+$ signalling is functional with an important role in CLL. RQ-PCR has shown increased Vimentin in patient samples. Immunoblotting identified Vimentin protein in CLL cell lines and UM-CLL patients, an interaction between Vimentin and pERK was demonstrated with Co-IP. IHC and fluorescent staining of cells demonstrated increased Vimentin levels in UM-CLL patients compared to M-CLL patients, also observed in CLL cell lines. Summary. Wnt16b appears to be the most consistently upregulated Wnt gene in CLL patient samples. Aberrant, non-canonical Wnt/$Ca2^+$ signalling potentially upregulates Vimentin expression. Vimentin expression is significantly greater in UM-CLL patients and UM-CLL cell lines at RNA and protein levels and can also be observed at greater levels microscopically throughout the cells. It appears that Vimentin and pERK interaction could potentially be sustaining activation of pERK signalling which may be contributing to the pathogenesis of CLL patients with unmutated IGHV.

0756

VIMENTIN EXPRESSION PREDICTS POOR PROGNOSIS IN CLL

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Background. Vimentin is an intermediate filament protein essential for lymphocyte rigidity and integrity. In solid tumors its high expression has been associated with metastatic and aggressive disease. In CLL, vimentin expression is heterogeneous and a high expression may indicate more resistant cells but its prognostic value is still undefined yet. *Aims.* 1) to evaluate vimentin expression in CLL patients; 2) to compare vimentin expression with other clinical and biological prognostic factors: Binet staging, IgVH status, expression of CD38 and Zap-70, VEGFR (VEGF receptor) and molecular cytogenetics (by FISH); 3) to evaluate the impact of vimentin expression in the progression free survival (PFS). *Methods.* Sixty-six patients with CLL were studied between September 2005 and October 2009. Flow cytometry analyses for CLL diagnosis (score system of Moreau *et al.*), CD38 and Zap-70 expression (fresh whole peripheral blood) and VEGFR and vimentin (mononuclear cells by Ficoll-Hypaque gradient method) were performed in fresh and/or cryopreserved blood samples at patient admission(figure). Vimentin expression was also evaluated in BM biopsy specimens obtained at diagnosis, by immunohistochemistry. The BM infiltration pattern was also evaluated. The IgVH genes mutational status was determined in mononuclear cells by RT-PCR followed by sequencing analysis. Molecular cytogenetics were characterized by fluorescence *in situ* using the 13q14.3 (D13S319), 13q34 (Proz, CUL4A, LAMP1), 17p13.1 (p53), 11q22 (ATM) and the chromosome 12 centromeric probe CEP 12 (D12Z3) (Vysis inc., USA). At least 200 interphase nuclei with well-delineated signals were counted in each slide. *Results.* Vimentin expression in both circulating and marrow cells was positively associated with unmutated IgVH genes, diffuse bone marrow infiltration, unfavorable cytogenetics abnormalities(del11q; del17p) and VEGFR positive expression. Vimentin expression in PB cells by flow cytometry and in BM cells by immunohistochemistry yielded concordant results, as did vimentin expression in fresh and thawed samples from the same patients. Association between vimentin expression and CD38 expression was observed only when vimentin was determined in BM. Advanced clinical stage, age<68 years, positive expression of CD38 and VEGFR, unmutated IgVH genes, unfavorable cytogenetics abnormalities, vimentin expression in PB and BM cells and diffuse marrow infiltration pattern were factors significantly associated with shorter PFS. The impact of these prognostic markers was also observed when only Binet A patients were analyzed. In multivariate analysis, the Binet's stages B+C (P=0.008), strong unimodal and bimodal MFI (mean fluorescence intensity) histogram pattern of CD38 (P=0.03), diffuse marrow infiltration (P=0.02) and vimentin expression on BM cells by immunohistochemistry (P=0.002) were identified as independent risk factor for reduced PFS. *Conclusions.* Vimentin expression in CLL cells from both PB and BM showed high impact as prognostic marker, predicting poor PFS, even in Binet A patients. Both flow cytometry and immunohistochemistry are relatively easy techniques and are feasible for most flow cytometry and pathology laboratories. Vimentin expression was comparable with IgVH mutational status suggesting its possible usefulness as prognostic marker in CLL patients. In addition, studying vimentin expression might also be useful for better understanding the pathophysiology of CLL and also for possible therapeutic interventions.

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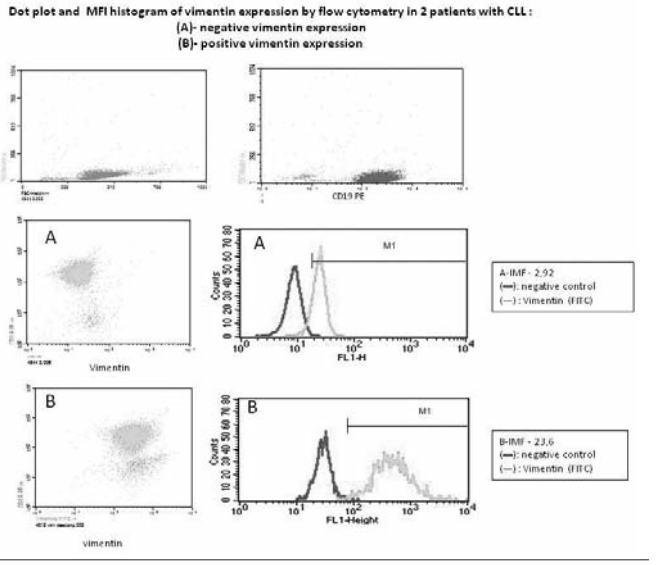

Figure 1.

0757

GENES ASSOCIATED WITH LIPID METABOLISM SEGREGATE WITH ZAP-70 EXPRESSION AND IGVH MUTATIONAL STATUS IN CLL PATIENTS

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Background. Chronic lymphocytic leukemia (CLL) is a heterogeneous disease. A relevant number of studies have shown IgVH mutation status and ZAP-70 expression as the most relevant prognostic markers in CLL, suggesting the separation of two patients subgroups with good (M IgVH ZAP-70^-), and poor prognosis (UM IgVH ZAP-70^+). *Aims.* Our aim was to determine gene expression profiles of 105 CLL patients divided into three classes: the first (n=61) with M IgVH and ZAP-70^-, the second (n=28) with UM IgVH and ZAP-70^+, and the third (n=16) included CLL patients with UM IgVH and ZAP-70^- or M IgVH and ZAP-70^+, respectively. Finally, the purpose was to identify molecular signatures underpinning the diverse clinical-biological phenotypes. *Methods.* We determined gene expression profiles using Affymetrix HGU133 Plus 2.0 in $CD19^+$ leukemic cells. A first cohort of 62 subjects was analyzed using GeneChip® Expression 3' Amplification One-Cycle Target Labeling kit, while a second cohort of 43 subjects using GeneChip® 3'IVT Express Kit. Differentially expressed genes were detected independently in the two cohorts using ANOVA and t-test adapted for microarray data analysis, and corrected for multiple testing using false discovery rate p-values. Only genes selected as differentially expressed in both datasets were considered for further analysis. To assess the association of the molecular signature based on the identified genes, the samples of five recently acquired CLL patients, not used for the differential analysis, were classified as belonging to class one or two, based only on their expression profile. Results. Statistical analysis revealed 42 differentially expressed genes in the first (M IgVH and ZAP-70^-) *vs.* the second (UM IgVH and ZAP-70^+) group. Interestingly, among them, ZAP-70 (zeta-chain TCR associated protein kinase 70kDa), LPL (lipoprotein lipase), MBOAT1 (membrane bound O-acyltransferase domain containing 1), P2RX1 (purinergic receptor P2X, ligand-gated ion channel, 1), DCLK2 (doublecortin-like kinase 2), and CHPT1 (choline phosphotransferase 1) were overexpressed, while ADAM29 (ADAM metallopeptidase domain 29) and NRIP1 (nuclear receptor interacting protein 1) were underexpressed in the first group with respect to the second. Notably, LPL, MBOAT1, CHPT1 are associated with lipid metabolism process. Cluster analysis (K-means, Euclidean distance) revealed that the 105

subjects are better partitioned in two rather than in three classes, based on their expression profiles. Based on the expression of the 42 genes previously identified, the 16 subjects of the third group were divided in two clusters: 12 more similar to group one, and 4 more similar to group two. Classification of five recently acquired patients, as belonging to class one or two based only on their expression profile, gave precision equal to 100%, thus confirming the molecular signature based on the identified genes. Conclusions. Our studies revealed that the well known LPL, MBOAT1 and CHPT1, associated with lipid metabolism, might be involved in CLL pathogenesis. Correlation of expression of the selected genes with ZAP-70, as well as IgVH and disease progression, will be evaluated to explore the potential value of these genes for future risk stratification. Moreover, functional investigations will be performed to provide new insight in the pathogenesis of CLL.

0758

CHRONIC LYMPHOCYTIC LEUKEMIC (CLL) B CELLS EXPRESS THE HGF RECEPTOR (C-MET) AND ARE SUPPORTED IN THEIR SURVIVAL BY HGF-PRODUCING MESENCHYMAL STROMAL CELLS

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Chronic lymphocytic leukemic (CLL) B cells are characterized by an apparent longevity *in vivo* but tend to undergo apoptosis when cultured *in vitro*. This evidence suggests that interactions and factors provided by the microenvironment may promote survival, proliferation and drug resistance of CLL B cells. Understanding CLL-stroma cross talk is actually of great interest to identify signals and molecules supporting leukemia expansion. Bone marrow (BM) is composed by different types of mesodermic cells, mesenchymal stem cells (MSC), differentiating along several lineages. Little is known on the effects exerted by undifferentiated/differentiated MSC on CLL and which cells resident in BM provide a permissive environment that is critical in prolonging survival of leukemic B cells and in mantaining their progenitors. Aim of this study was to determine whether different mesenchymal stromal cell lineages could differently affect CLL survival and to identify growth factors, surface markers and signals involved in mantaining CLL expansion. We utilized bone marrow stromal cells (BMSC) and some different lineages of mesenchymal origin to investigate how mesenchymal stromal cells support CLL cells survival. We determined that different lineages of mesenchymal origin differently affect CLL B cells viability in co-cultures: human BMSC, fibroblasts or cells from trabecular bone significantly prolonged survival of leukemic B cells, while endothelial cells and chondrocytes did not. Moreover supportive effects were also mantained, although to a lesser extent, when leukemic B cells and stromal cells were separately cultured in transwell plates or when only conditioned medium, from different MSC lineages, were added in CLL cultures. Gene expression profiles comparison of different mesenchymal cells suggested two soluble factors, CXCL12 and HGF, as potential candidates involved in enhancement of leukemic B cells viability. In agreement with gene expression analysis we thus demonstrated that CXCL12 and HGF were produced only by mesenchymal lineages sustaining CLL survival and that CLL B cells express the HGFreceptor c-MET, in addition to CXCR4. Furthermore recombinant HGF, when added in *in vitro* cultures, is capable of increasing CLL viability and of inducing STAT3 phosphorylation. In conclusion we demonstrated that BMSC and some differentiated cells of mesenchymal origin prolong CLL survival and that two soluble factors, CXCL12 and HGF, are produced by those MSC capable of sustaining survival of leukemic B cells. Our study further underlines that HGF, together with CXCL12, can be pivotal in mediating resistance to apoptosis of CLL B cells, c-MET$^+$/CXCR4$^+$, at the bone marrow level. Moreover the *in vitro* culture systems that we here designed will be helpful to reveal more insights reflecting *in vivo* mechanisms related to disease progression and to identify novel therapeutic targets.

0759

CD38 AND CD49D ARE PHYSICALLY ASSOCIATED AND FUNCTIONALLY COOPERATING ON B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA CELL MEMBRANE

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Background. CD38 and CD49d are associated negative prognosticators in chronic lymphocytic leukemia (CLL) with a direct role in the interactions of CLL cells with other microenvironmental cell populations. *Aims.* The propensity of CD38 and integrins to form supramolecular complexes, prompted us to investigate whether specific CD49d and CD38 interactions occur on the CLL membranes, and whether the resulted molecular complexes may have a role explaining the aggressiveness of CD38/CD49d-expressing CLL. *Methods.* Confocal microscopy and biochemical approaches were used to study the membrane organization of CD49d and CD38 in primary CLL cells and other B cell lines. The CLL-derived CD49d+CD38$^-$ cell line Mec-1 (Mec-1-CD38$^-$) was used as a model in adhesion experiments. Viral transduction was used to induce CD38 expression by Mec-1 (Mec-1-CD38$^+$). Apoptosis was investigated by syto-16/7-AAD staining in flow cytometry. *Results.* Co-capping experiments in CLL cells demonstrated a membrane relationship between CD38 and CD49d. Anti-CD49d monoclonal antibodies (mAbs) induced capping in approximately 75% of CLL cells, with a 80% redistribution of CD38 in the context of the capping area. Similar results were obtained with the cell lines Raji and RPMI-8226, both constitutively expressing high CD38 and CD49d levels. The CD38/CD49d lateral association was confirmed at the biochemical level by co-immunoprecipitation experiments with anti-CD49d mAbs and subsequent blotting of immunoprecipitates using anti-CD38 mAbs. CD38-CD49d association was also maintained after engagement of CD49d with its natural ligands, as witnessed by a striking co-localization of CD49d and CD38 in cell uropods formed by CLL and Raji cells adhered and spread onto VCAM-1 and CS-1 fibronectin (FN) fragments. Notably, these experiments also highlighted the involvement of specific CD38 domains in the CD38/CD49d/VCAM-1 molecular complex, as evidenced by staining with anti-CD38 antibodies recognizing different epitopes. To investigate whether the CD38/CD49d association had also a functional meaning, adhesion assays on VCAM-1-coated plates were performed with the Mec-1 cell model. Mec-1-CD38$^+$ showed a marked increase in VCAM-1 adhesion compared to Mec-1-CD38- (mean values of adhered cells relative to control=5.4 *vs.* 2.3 and 5.4 *vs.* 1.9 after 15 and 30 minutes respectively). Moreover, phase-contrast and immunofluorescence microscopy highlighted clear differences in the morphology of adhered cells, with Mec-1-CD38$^+$ cells characterized by a more complex pattern of uropods than Mec-1-CD38$^-$, and a clear colocalization of CD38 and CD49d in adhesion sites. Finally, we tested whether CD38 engagement could have a role in enhancing the protection against spontaneous apoptosis of CLL cells cultured onto CD49d ligands. Purified CLL cells (n=6) were cultured in the presence of anti-CD38 mAb or CS-1 FN fragment, either alone or in combination. Analysis of cell apoptosis after a 72-hour culture, showed 79±1.1% viable cells after CD49d independent engagement, and a substantial improvement of cell viability (94%±0.5) after exposure to both anti-CD38 mAb and CS-1 fragment ($P<0.01$). *Conclusions.* CD49d and CD38 are physically associated on CLL cell membranes. Such a physical association influences the anti-apoptotic cell adhesion process, suggesting a cooperative role for CD38 and CD49d which may explain at least in part the poor clinical outcome of the disease subset co-expressing these molecules.

0760

SYNERGISTIC ACTIVATION AND INCREASED MIGRATION UPON CCL21 ADDITION IN ABNORMAL ZAP-70 EXPRESSING B CELLS

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Background. ZAP-70 (ξ-associated protein) is a protein tyrosine kinase (PTK) of the Syk/ZAP family normally expressed in T and NK cells.

When the TCR homodimer transmits the extracellular binding event to the cytoplasma via the immunoreceptor tyrosine-based activation motifs (ITAMs), ZAP-70 protein binds to the double phosphorylated ITAMs. This initiates downstream biochemical events that lead to T-cell proliferation and differentiation. Recent studies have showed that ZAP-70 protein is also expressed in leukemic B cells. Increased ZAP-70 expression in Chronic Lymphocytic Leukemia (CLL) correlates with unmutated IgVH genes, a short time of progression, and a short survival. Mechanisms by which increased ZAP-70 protein expression can influence clinical outcome are not fully understood. Objectives: To study the functional consequences of ZAP-70 exogenous expression and activation in B-cell lymphoma cell lines. *Methods.* Ramos and Raji (Burkitt) cell lines were stably transfected with the ZAP-70 expressing vector pEGFP-N2ZAP-70. Western Blot was used for protein studies. ZAP-70 expression, cell cycle, IgM expression, calcium mobilization, surface molecules and migration were analyzed by flow cytometry. *Results.* Upon BCR engagement simultaneous phosphorylation and activation of ZAP-70 and Syk protein (the equivalent of ZAP-70 in B cells) was observed in Ramos cell line. Interestingly, a constitutive activation of ZAP-70 was observed in Raji cell line. In addition, Erk and Akt proteins were strongly activated this lasting more than 24 hours. This enhanced signalling may be due the observed reduction of BCR internalization after antigen ligation in the ZAP-70 positive B cells. ZAP-70 phosphorylation led to increased percentage of cells in S and G2/M cell cycle phases and an expression of higher numbers of surface molecules, being chemokine receptor CCR7 the more remarkable one. CCR7 increased expression after ZAP-70 activation had a direct positive effect on migration upon CCL21 addition (CCR7 ligand). We observed that CCL21 chemokine also induced activation of Akt protein in ZAP-70 expressing cells. In this regard, after simultaneous BCR and CCR7 stimulation, an additive Erk and Akt phosphorylation was observed. Furthermore, when adding CCL21 after IgM activation an increased and prolonged mobilization of $Ca2^{+}$ was observed in ZAP-70 positive cells, probably due to the increased CCR7 expression induced by BCR stimulation. *Conclusions.* Heterologous ZAP-70 expression into a B-cell system enhances BCR downstream signalling not only through conventional IgM activation but also through CCR7 signaling. Therefore, CCR7 overexpression in ZAP-70 activated B cells, upon CCL21 chemokine addition, induces not only a synergistic activation but also an increased calcium mobilization and migration of the cells. It is important to highlight that CCL21 is expressed in the lymph nodes where ZAP-70 positive cells may have an increased propensity to migrate and the ability to respond to survival signals. Althogether, these data suggest that ZAP-70 expression in CLL and other lymphoproliferative disorders enhances tumoral activity, this explaining in part the poor clinical prognosis of patients with increased expression of ZAP-70.

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SMALL CLL-LIKE B-CELL CLONES IN HEALTHY ADULT SUBJECTS: LEUKEMIC PRECURSORS VERSUS COUNTERPART OF CLL CELLS?

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Background. Chronic lymphocytic leukemia (CLL) is the most common subtype of leukemia in Western countries. In the last decade, it has been reported that circulating CLL-like B cells can be detected at low levels (<5×10^{9}/L) in healthy subjects, this being termed "Monoclonal B-cell lymphocytosis"(MBL), independently of the presence *vs.* absence of lymphocytosis. Although initially the presence of CLL-like B-cell clones was reported to be around 3-6% of healthy adults (>40 years), the use of more sensitive flow cytometry approaches has shown that the actual frequency could be near three times more in healthy adults >40 years. These recent findings prompted us to question about the pathological *vs.* physiological nature of B-cell clones in normal subjects. *Aim.* To estimate whether the frequency of cases in which a CLL-like B-cell clone is detected raises to 100% if higher volumes of peripheral blood (PB) are screened. *Methods.* A total of 639 healthy individuals (46%males / 54%females) >40 years old (62±13 years) with normal lymphocyte counts ($2.1\pm0.7\times10^{9}$/L) were selected across the Primary Health Care area of Salamanca (Spain). Between 0.9-1.2 mL/tube EDTA-anticoagulated PB were immunophenotyped using a high-sensitive flow cytometry approach, based on 8-color staining panels and the systematic screening for >5×10^{6} total PB leucocytes. The minimum number of cellular events required to define a CLL-like B-cell cluster was of 50 cells. In each case, the real volume of blood screened was registered, and in MBL cases, the actual volume of PB containing the first 50 CLL-like B-cell events was calculated. A statistical predictive model (power regression, using the MATLAB software) was built, to estimate for the whole cohort of individuals and for each age-group (40-59, 60-69 and >70 years), the percentage of subjects showing CLL-like cells, in the hypothetical case that higher volumes of PB would be stained. *Results.* Overall 12.5% of cases carrying CLL-like B-cell clones was detected after measuring 0.9 to 1.2 mL of PB. A progressively higher frequency of cases carrying CLL-like B-cell clones was observed in parallel to increasing age. Our predictive model suggested that, if larger volumes of PB (e.g. ≥50 mL) were analyzed, 100% of healthy adults over 70 years may carry detectable CLL-like clones (the lower limit of the 95% confidence interval -CI- reached 100% for PB volumes of ~46 mL). According to this model, in the hypothetical case that 50 mL PB would be stained per case, the estimated frequency of cases carrying CLL-like clones for the 40-59-year group was of 32% (95% CI: 18% to 46%) and of 62% for cases with 60-69 years (95% CI: 36% to 88%); with an estimated rate of 100% for the whole series (lower limit of the 95% CI of 70%). Summary/*Conclusions.* Altogether, these results support the notion that over a certain age, the emergence of one or more CLL-like clones would probably occur, suggesting that these clonal cells may more likely represent the normal counterpart of CLL cells, rather than leukemic precursors. Further studies are necessary to confirm this hypothesis.

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CD69 AND CD79B OVEREXPRESSION IDENTIFY POOR RISK CHRONIC LYMPHOCYTIC LEUKEMIA

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Leukemic cells from all chronic lymphocytic leukemia (B-CLL) cases exhibit features of activated and antigen-experienced B-lymphocytes and up-regulation of CD69 is more frequent within unmutated or CD38 positive CLL subsets (Damle, 2002 and 2007). Moreover, high B-cell receptor (BCR) signaling characterized by CD79b overexepression increases cell survival and cell cycle progression. In fact, CD79b has been preliminarly correlated with atypical morphology, unmutated IgVH status and short overall survival (OS) (Zucchetto, 2006). Therefore, a hyperactivated and BCR hyperstimulated B-CLL phenotype could allow us to identify a subset of patients who would have a more aggressive disease. The primary endpoints of our research were: 1) to determine progression free survival (PFS) and OS upon CD69 and CD79b expression; 2) whether CD69 and CD79b expression had additive prognostic value and finally 3) whether CD69 and CD79b were independent prognostic factors in multivariate analysis. We investigated 412 patients, median age 65 years (range 37-87), 219 males and 193 females. With regard to modified Rai stages, 124 had a low stage, 270 an intermediate stage and 18 a high stage. CD69 and CD79b were determined by multicolor flow cytometry, using L78 and SN8 clones, respectively, and fixing the cut-off value at 30%. $CD69^{+}$ and $CD79b^{+}$ B-CLL patients were 108/401 (27%) and 208/402 (52%), respectively. CD69 and CD79b >30% were significantly associated with intermediate/high Rai stage (P<0.0001), lymphocyte doubling time <12 months (P<0.0001), beta-2 microglobulin >2.2 mg/dL (P<0.0001) and soluble CD23 >70 u/mL (P<0.0001). There were significant correlations between CD69 <30% and normal or del13q FISH cytogenetics (296 cases studied, P<0.0001). Noteworthy, there was a strict correlation either between CD79b >30% and trisomy 12 (25/33; P=0.001) or CD79b >30% and del17p (16/20; P=0.001). There were significant correlations between lower CD69 (226 cases studied, P=0.0007) or lower CD79b (253 cases studied, P<0.00001) and IgVH gene mutated status. Equally, significant associations were found between ZAP-70, determined by flow cytometry, and CD69 (P=0.008) or CD79b (P<0.0001). With regard to clinical outcome, both shorter PFS and OS were observed in $CD79b^{+}$ patients (10% *vs.* 56% and 40% *vs.* 93% at 14 years, P<0.00001) as well as in $CD69^{+}$ patients (3% *vs.* 49% at 14 years, P<0.00001 and 44% *vs.* 67% at 14 years, P=0.00004). Noteworthy, CD79b and CD69 showed additive prognos-

tic properties, since CD79b <30% plus CD69 <30% identified a B-CLL subset at better prognosis with regard to PFS (77% *vs.* 2% at 14 years; P<0.00001) and OS (95% *vs.* 31% at 14 years; P<0.00001). The two discordant subsets ($CD69^+CD79b^-$ and $CD69^-CD79b^+$) showed an intermediate outcome (Figure). In multivariate analysis of PFS and OS, in which entered age, Rai modified stages, ZAP-70, lymphocyte doubling time, beta-2 microglobulin, CD38, CD69 and CD79b, both CD69 (P=0.008 and P=0.002) and CD79b (P=0.0003 and P=0.0006) resulted to be independent prognostic factors. Therefore, CD69 and CD79b, determined by flow cytometry, should be considered novel important prognostic parameters in B-CLL. Their easy and rapid laboratory evaluation could allow us to identify early progressive patients taking timely therapeutic decisions.

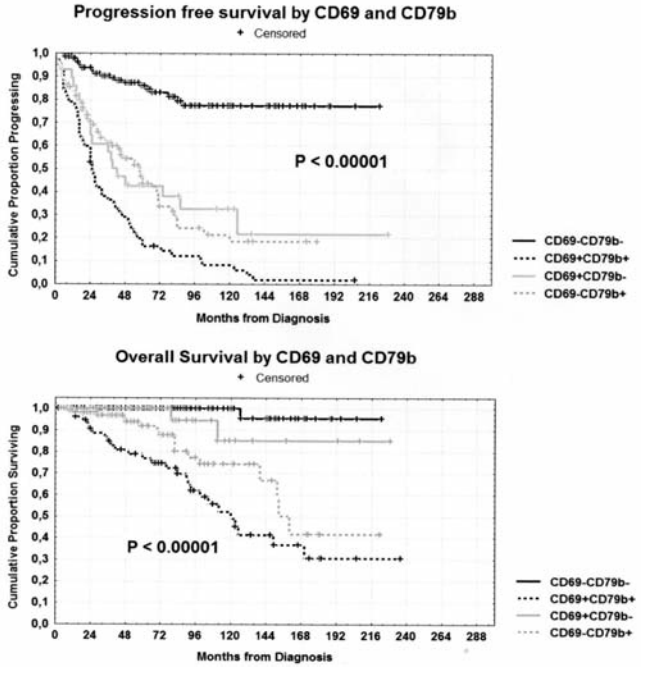

Figure 1. Clinical outcome by CD69 and CD79b.

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ASSOCIATION STUDY OF KILLER CELL IMMUNOGLOBULIN-LIKE RECEPTOR GENES AND HUMAN LEUKOCYTE ANTIGEN CLASS I LIGANDS WITH B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA IN THE POLISH POPULATION

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Introduction. B-cell chronic lymphocytic leukemia (B-CLL) patients have several humoral and cellular abnormalities. It has been suggested that innate and immunity, especially natural killer (NK) cells, and subpopulations of T-cells play a key role in antitumor cytotoxicity regulated by the interaction between killer immunoglobulin-like receptors (KIRs) and their HLA-class I ligands on target cells. KIR receptors may be either inhibitory, with long cytoplasmic tails (DL), or activating, with short cytoplasmic tails (DS). 2DL2, 2DL3, and 2DS2 KIRs bind to the HLA-C1 allotype (Asp80), while the 2DL1 and 2DS1 KIRs bind to the HLA-C2 allotype (Lys80). There is strong evidence that polymorphisms in KIR genes and their cognate HLA ligands contribute to the pathogeneses of several diseases, including infections, autoimmunity, and, recently, cancer, but literature data for B-CLL are inconsistent and limited. *Aim.* The aim of this study was to investigate the association between polymorphism of KIR genes and their HLA-ligands and susceptibility to B-CLL. *Materials and methods.* The KIR genes 2DL1, 2DL2, 2DL3, 3DL1, 2DS1, 2DS2, 2DS3, 2DS4full length , 2DS5, 3DS1, and 2DS4with a deletion were genotyped in 197 B-CLL patients and in 200 controls by PCR-SSP methods. The HLA-C1, HLA-C, HLA-Bw4(Thr80), and HLA-Bw4(Iso80) specificities were typed in 185 B-CLL patients and 200 healthy individuals using an Olerup SSP typing Kit. *Results.* The frequencies of the individual KIR genes did not differ in the two studied groups, but a trend toward a lower frequency of KIR2DS3 gene in the B-CLL patients compared with controls (26.9% *vs.* 35.5%, P=0.065, OR: 0.67, 95%CI: 0.44-1.03) was noted. There were no statistically significant differences in haplotype and genotype frequencies; however, the higher frequency of individuals possessing genotypes with prevalanceof inhibitory over activating KIR genes in the B-CLL patients compared with the controls was observed (ratio of activating to inhibitory KIR genes between 0.2-0.83: 83.2% *vs.* 75.5%, P=0.06, OR: 1.6, 95%CI: 0.98-2.64), while healthy individuals were overrepresented in the group of genotypes with a prevalence of activating KIR genes (activating/inhibitory KIR gene ratio: 1- 1.5). Analysis of the distributions of HLA-C1 and HLA-C2 allotypes and genotypes in the B-CLL patients and controls did not reveal any differences, but the frequency of HLA-Bw4 specificity was significantly reduced in the B-CLL patients compared with the controls (48.6% *vs.* 63.0%, P=0.0045, OR: 0.56, 95%CI: 0.37-0.84). Moreover, there were fewer individuals possessing HLA-Bw4 with threonine in the B-CLL group, especially those having HLA-Bw4(Thr80) and lacking HLA-Bw4(Ile80) (21.6% *vs.* 32.0%, P=0.022, OR: 0.59, 95%CI: 0.37-0.93 and 16.2% *vs.* 26.5%, P=0.014, OR: 0.54, 95%CI: 0.32-0.89). 3S1+/HLA-$Bw4^+$ and $3DL1^+$/HLA-$Bw4^+$ combinations were consequently less common in the B-CLL patients than in the controls (14.6% *vs.* 27.0%, P=0.0028, OR: 0.46, 95%CI: 0.28-0.77 and 45.4% *vs.* 57.0%, P=0.023, OR: 0.63, 95%CI: 0.42-0.94), particularly those with the coexistence of 3DL1, 3DS1, and HLA-Bw4 (12.4% *vs.* 21.0%, P=0.025, OR: 0.53, 95%CI: 0.31-0.93). *Conclusions.* These results indicate that genotypes with a prevalence of inhibitory over activating KIR genes confer susceptibility to B-CLL while the presence of activating KIR2DS3 and HLA-Bw4 might be associated with protection from it.

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MALDI-TOF MS PROFILING IDENTIFIES ION SIGNALS DIFFERENTLY EXPRESSED IN PROGNOSTICALLY DEFINED SUBSETS OF BINET STAGE A B-CELL CHRONIC LYMPHOCYTIC (CLL) RECOGNIZED BY BIOLOGICAL MARKERS

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Background. CLL is characterized by the expansion of monoclonal mature B lymphcytes with an extremely heterogeneous clinical course. Proteomic analysis is particularly attractive to provide insights into the biological complexity of CLL and can be used to identify ion signals which are differently expressed in cases exhibiting markers of well-known prognostic impact, including CD38 and ZAP-70 expression, and IgVH gene mutational status. Patients and Methods. A panel of highly purified neoplastic cells (>92%) from 42 untreated, newly diagnosed CLL patients in Binet stage A was investigated. We used proteomic technology, MALDI-TOF MS profiling to examine the protein content of CLL cells. Highly purified neoplastic B lymphocytes were subjected to cell lysis, followed by sub-fractionation of the water-soluble component. An aliquot of this fraction underwent a desalting/concentration step over ZipTip C18 and peptide/protein profiles were analysed using a VoyagerDE PRO MALDI-TOF mass spectrometer (PerSepyiveBiosystem). Separate spectra were obtained for a restricted mass-to charge (m/z) range (1000-25000 Da) in linear mode geometry, by applying an accelerated voltage of 25 kV. The acquired spectra, assayed in duplicate, were then processed for automated advanced baseline correction and noise. The peak area of each signal was normalized as a percentage of the total peak area (individual peak area/total area per cent). Supervised analyses for CD38 and ZAP-70 expression, and IgVH mutational status were performed, and the significance of ion signals differently expressed were evaluated by student's t-test. Results. Supervised analysis allowed the identification of 23, 32 and 28 differentially expressed ion signals with statistical significance (P<0.05), respectively in CD38 (14 positive), ZAP-70 (13 positive), and IgVH (12 unmutated) . Comparing differentially expressed ion signals, 6 m/z values (2162,9; 2782,5; 3110,5; 4572,3; 6973,6; 10463,2 m/z values) were shared by the three ion signal lists. A supervised analysis was also conducted using a recently published prognostic score system (Morabito F *et al.*, BJH, 2009). Comparing cases scored 3 (i.e. CD38-positive, ZAP-

70-positive and unmutated-IgVH) with those scored 0 (i.e. with no negative prognostic marker), we have identified 25 differentially expressed ion signals with statistical significance (P<0.05), among which we recognized the same above mentioned 6 ion signals. Conclusion. These results demonstrate that the B-CLL neoplastic cells may be classified on the basis of their protein/peptide content using the MALDI-TOF profiling analysis. Future studies will focus on the identification of the differentially expressed ion signals.

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A PROTEOMIC STRATEGY IDENTIFIED ELEVATED LEVELS OF SERUM AMYLOID A (SAA) PROTEIN IN THE PLASMA OF CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

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Background. Chronic Lymphocytic Leukemia (CLL) is the most common leukemia in the western world and is characterized by the accumulation of relatively mature B cells. CLL is notable for variation in aggressiveness of disease. Many patients with low-risk disease at diagnosis can be followed expectantly, while others rapidly require therapy. This clinical heterogeneity appears to be sustained by different biologic parameters, such as the immunoglobulin variable gene (IgVH) mutational status, ZAP-70 expression and specific cytogenetic alterations. Many patients express a combination of all favorable or all unfavorable markers, but there are also patients with different marker combinations. Unique easily accessible prognostic markers helping to predict the individual course of CLL are therefore highly warranted. *Aim of the study.* To use a proteomic strategy to identify differentially expressed proteins in the plasma of CLL patients. *Methods.* Proteomic profiling of plasma or serum is a technique to identify new biomarkers in disease. The objective of this study was to identify new plasma biomarkers in CLL patients using surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometry. To first establish the feasibility and specificity of the method using a case-control study design, plasma samples from 30 patients with a known CLL diagnosis were compared to samples from 30 age- and sex-matched healthy controls. All samples were run in triplicate and the obtained spectra aligned. Protein spectra were generated and the protein peak intensities normalized to the total ion current m/z values and analysed using Ciphergen ProteinChip software 3.2.0. Comparison of SELDI profiles using the Unsupervised Cluster analysis highlighted that the plasma profiles of patients with CLL could be clearly separated from control groups. Application of the BMV Program to these spectra identified the m/z 11,681 cluster as most significantly different. Preparative monodimensional electrophoresis was used to isolate this cluster from plasma that highly expressed or did not express the m/z 11,681 cluster. MALDI-TOF MS analysis identified the unknown protein as Serum amyloid A (SAA). We have previously demonstrated that plasma SAA SELDI-TOF intensities are correlated with SAA protein concentrations (r=0.41; P=.0001) determined by ELISA. Thus, plasma SAA concentrations were measured by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (Biosupply Ltd., Bradford, United Kingdom) in a larger group of 86 newly diagnosed B-CLL patients (44 males, 42 females median age of 62 years) and 30 age- and sex-matched healthy controls. *Results.* The analysis revealed statistically significant differences in SAA expression between the plasma of LLC patients and the plasma of healthy subjects (P=0.002). In addition, SAA levels are elevated in the plasma of patients with an unfavourable cytogenetic profile (try12; del17; del11) when compared to patients with normal or other cytogenetic status (P=0.02). In patients, SAA plasma levels are also positively correlated with peripheral lymphocyte doubling times of less than one year (P=0.009). *Conclusions.* Although preliminary, these data suggest that elevated SAA levels are associated with unfavorable known prognostic markers and support the view that inflammation is implicated in CLL development.

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GENETIC VARIATION IN TNF AND IL-10 AND CLINICAL OUTCOME OF B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Recent studies have demonstrated that common genetic variants in immune and inflammatory response genes can play the important role in lymphomagenesis. TNF and IL-10 are good candidate genes since they code critical cytokines regulating the balance between the T-helper, Th1 and Th2, immune responses. Therefore we tested the hypothesis that single-nucleotide polymorphisms (SNPs) in TNF and IL-10 promoters influence susceptibility and clinical course of B-cell chronic lymphocytic leukemia (CLL). Patients and *Methods.* We genotyped TNF -308G>A (rs1800629), IL-10 -1082A>G (rs1800896) and IL-10 -3575T>A (rs1800890) in 190 newly diagnosed patients with CLL and 192 ethnically-matched healthy individuals using the Taqman™ platform (Applied Biosystems, USA). Some randomly selected DNA samples were analysed by direct sequencing using 3130xl Genetic Analyzer (Applied Biosystems, USA). Haplotype analysis was performed using version 2.0.2 PHASE software. The IgVH mutation status in CLL patients was performed according to the protocol described by van Dongen *et al.* (Leukemia 2003). *Results.* The TNF -308G>A, IL-10 -1082A>G and IL-10 -3575T>A allelic frequencies and distributions were consistent with Hardy-Weinberg equilibrium, and did not differ significantly between CLL patients and the control group. There were no significant differences in estimated frequencies of the IL-10 haplotypes between CLL patients and controls. No association was found between TNF and IL-10 allelic, genotype or haplotype distributions and clinical characteristics of CLL patients at diagnosis, including age, clinical stage according to Rai classification, serum LDH and β2-microglobulin levels, as well as surface CD38 expression, ZAP-70 expression, and IgVH mutation status. The patients with IL-10(-1082G) allele (IL-10-1082GG or IL-10-1082GA genotypes) had significantly shorter time from diagnosis to treatment (11 months *vs.* 24 months, P=0.02, Mann-Whitney test) as compared to individuals carrying IL-10(-1082AA) genotype. Neither of assessed TNF and IL-10 SNPs was associated with response to first-line treatment or progression free survival. With a median follow-up of surviving patients of 58 months (range 1-209 months), the subgroups of patients with TNF(-308A) allele (TNF-308AA or TNF-308AG genotypes) had significantly shorter overall survival (OS) compared to those carrying TNF(-308GG) genotype (P=0.01, log-rank test). No correlations were found between IL-10 alleles, genotypes or haplotypes and OS in CLL patients. To further characterize the prognostic impact of TNF and IL-10 SNPs on CLL patients survival, we divided the patients according to the IgVH mutation status into a IgVH mutated (homology <98%) and IgVH unmutated (homology ≥98%) group. With respect to TNF SNP, the patients carrying TNF(-308A) allele presented significantly shorter OS both in the IgVH mutated and unmutated group compared to those with TNF(-308GG) genotype (P=0.02 and P=0.03, respectively). When IL-10 SNPs were analysed, the IL-10(-1082G) or IL-10(-3575A) alleles retained their prognostic impact on shorter OS only in the IgVH mutated group compared to the IL-10(-1082AA) or IL-10(-3575TT) genotypes (P=0.05 and P=0.02, respectively). *Conclusions.* Our results indicate that TNF and IL-10 gene variations may influence CLL outcome especially in the IgVH mutated subgroup, which points out the importance of innate immunity genes for CLL prognosis.

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AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION (AUTOHSCT) IN CLL: FIRST RESULTS OF AN EBMT RANDOMIZED TRIAL COMPARING AUTOTRANSPLANT VERSUS WAIT AND WATCH

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Material and *Methods.* This phase-III randomized EBMT-intergroup trial studied the impact of a consolidating autoHSCT *vs.* wait and watch for patients with CLL in Binet stage A progressive, B or C , in CR, nodular PR or VGPR after first or second line therapy. *Aim.* The primary objective was to show that autoHSCT increase the 5-year progression-free survival (PFS) by 30%. Here we present a first analysis based on 80% of expected follow-up forms. *Results.* Between November 2001 and July 2007, 223 patients were enrolled (SFGM-TC/FCLLG n=99, MRC n=63, GCLLSG n=36, SAKK n=10, other EBMT centers n=15). There were 74% males and 26% females. Binet stages were progressive A 13%, B 67%, C 20%; 59% were in CR, and 41% in very good or nodular PR. Of note, SFGM-TC/FCLLG included only patients in CR. Eighty three percent of the patients were enrolled in 1st, and 17% in 2nd line treatment. Patients were randomized between group 1 (autoHSCT n=112) and group 2 (observation n=111) after an induction treatment which was left at the discretion of the investigators. Median PFS was 26.6 months (18.3-35) in the observation group and 50.1 months (40-60.1) in the autoHSCT group; the 5-year PFS was 29% and 42%, respectively (P<0.001). Accordingly, the 5-year relapse incidence was 54% *vs.*70%.; P<0.001. The Cox modeling for randomization arm, Binet stage, disease status, line of treatment, contributing group (country), and the interaction between randomization arm and contributing group confirmed that autoHSCT significantly improved PFS (HR 0.45 [0.30-0.66] P<0.001). The beneficial effect of autoHSCT was stable over all contributing groups. At 5 years, the probability of OS was 83% and 82% for autoHSCT and observation, respectively; P=0.81. Significant differences in terms of non-relapse death were not observed. *Conclusion.* in patients with CLL in first or second remission, consolidating autoHSCT reduces the risk of progression (PFS) by more than 50%, but has no effect on overall survival. There was no significant difference of non-relapse deaths in the autograft arm compared with the watch and wait arm. Further analyses on variables affecting the outcome are underway and results from a quality of life study on both groups are awaited.

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FLUDARABINE (FLU) PLUS ALEMTUZUMAB (FLUCAM) IMPROVES PROGRESSION FREE SURVIVAL VERSUS FLUDARABINE IN PREVIOUSLY TREATED CHRONIC LYMPHOCYTIC LEUKEMIA AND DEMONSTRATES ACTIVITY IN HIGH RISK PATIENTS

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Background. After the phase II study of FluCam in previously treated CLL patients demonstrated meaningful efficacy with acceptable safety (Elter JCO 2005), this phase III prospective, multicenter, open-label, randomized, controlled study was conducted. *Aims.* We report updated efficacy and safety results along with subgroup analysis by adverse prognostic factors (PF) including age ≥65 years. *Methods.* Treatment arms were balanced by study center, Rai stage, disease status, age, gender, prior Flu therapy, and maximum lymph node (LN) size. During cycle 1, FluCam patients received escalating doses of Cam IV until the 30 mg dose was tolerated. Then, they received Flu 30 mg/m^2 IV followed by Cam 30 mg IV days 1-3 every 28 days. In the Flu arm, patients received 25 mg/m^2 IV days 1-5 every 28 days. Patients received up to 6 cycles. The primary endpoint was progression-free survival (PFS), secondary endpoints included overall response (OR), complete response (CR), overall survival (OS) and safety. Efficacy analyses were based on assessment by independent review panel. *Results.* Among 335 randomized patients (median age 60 years, range, 33-80), 19% had maximum LN size ≥ 5 cm, 37% were Rai Stage III-IV, 15% had prior Flu exposure, and 35% were ≥ 65years old. The median number of cycles received was 6 for both arms. The median PFS for FluCam was significantly longer compared with Flu (24.1 *vs.* 15.4 months, respectively, P=0.004; HR 0.64 [95% CI: 0.48-0.87]). Superior PFS with FluCam was also observed in patients ≥ 65 years, Rai Stage III-IV, and other adverse PF (Table 1). OR rates and CR rates for FluCam and Flu were 80.4% *vs.*74.3% (P=0.186) and 12.5% *vs.* 2.4% (P<0.001) respectively. No differences in OS have been observed, however, Rai III-IV patients receiving FluCam had significantly improved OS (P=0.005; HR 0.44 [95% CI: 0.24-0.79]). Adverse reactions occurring in >10% in the FluCam arm compared with Flu included pyrexia, leukopenia, chills, lymphopenia, urticaria, infusion related reactions, and rash. Incidence of grade 3/4 thrombocytopenia, neutropenia and anemia for FluCam and Flu were 17.7% *vs.* 22.7%, 63.7% *vs.* 68.9%, and 13.4% *vs.* 22.0%, respectively. Incidence of serious adverse events (SAEs) in FluCam was 32.9% and 25.5% for Flu. The most commonly reported SAE's were neutropenia and febrile neutropenia, with no difference in the frequency of these events (4.9% *vs.* 2.4% and 3.0% *vs.* 4.2% respectively). Symptomatic CMV infection occurred in 2.4% of patients, with 1.2% considered SAEs and none ≥ grade 4. The adverse reaction profile of FluCam in patients ≥ 65 years was comparable to the overall patient population. *Summary/Conclusions.* These data demonstrate that the FluCam combination is superior to Flu in previously treated CLL, including the elderly and advanced stage disease. FluCam demonstrated significantly longer PFS and a comparable safety profile with improved overall survival in Rai III-IV patients. FluCam shows an improved risk/benefit ratio compared to Flu and is an important option for previously treated CLL.

Table 1. Progression free survival and objective responses.

Demographics			Progression Free Survival (months)				Response Rates			
Group	FluCam N=168 N(%)	Flu N=167 N (%)	FluCam Median (95% CI)	Flu Median (95%CI)	p*	Hazard Ratio (95% CI)		FluCam %	Flu %	p†
Overall	168 (100)	167 (100)	24.1 (19.9, 28.4)	15.4 (12.2, 21.2)	0.004	0.64 (0.48, 0.87)	CR+PR	80.4	74.3	0.186
							CR	12.5	2.4	<0.001
Rai III-IV	61 (36)	62 (37)	24.5 (16.2, 31.9)	11.8 (9.3, 14.7)	<0.001	0.46 (0.29,0.73)	CR+PR	77	56.5	0.016
							CR	16.4	3.2	0.014
Age ≥ 65	57 (34)	60 (36)	26.2 (20.5, 37.8)	14.7 (11.8, 25.5)	0.011	0.49 (0.28, 0.86)	CR+PR	80.7	73.3	0.221
							CR	8.8	3.3	0.248
β2M ≥ 4mg/L	57 (34)	48 (29)	19.2 (13.9, 26.2)	11.8 (9.3, 14.7)	0.005	0.47 (0.28, 0.81)	CR+PR	73.7	64.6	0.334
							CR	7.0	2.1	0.231
Zap 70 ≥ 20%	68 (40)	72 (43)	26.6 (20.5, 42.3)	17.3 (11.8, 24.0)	0.015	0.55 (0.33,0.90)	CR+PR	82.4	75.0	0.186
							CR	13.2	1.4	0.006
CD 38 ≥ 30%	65 (39)	71 (43)	20.5 (18.6, 28.4)	13.6 (10.9, 21.2)	0.016	0.57 (0.36, 0.91)	CR+PR	78.5	73.2	0.263
							CR	12.3	1.4	0.013
Lymph Node ≥ 5 cm	34 (20)	31 (19)	18.7 (9.6, 23.9)	15.4 (11.9, 20.8)	0.639	0.88 (0.47, 1.65)	CR+PR	61.8	87.1	0.021
							CR	8.8	3.2	0.347

0769

CHEMOIMMUNOTHERAPY WITH OFATUMUMAB, FLUDARABINE AND CYCLOPHOSPHAMIDE IN PREVIOUSLY UNTREATED PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA: A PHASE II INTERNATIONAL TRIAL

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Introduction. Chemoimmunotherapy with CD20 monoclonal antibody (mAb) is the standard frontline treatment for physically fit patients with chronic lymphocytic leukemia (CLL). Ofatumumab is a

human mAb that binds a unique epitope encompassing both the small- and large-loop on CD20. The recent international pivotal study of single-agent ofatumumab demonstrated high overall response rates (ORR) of 47-58% in patients with fludarabine-refractory CLL. *Aims.* We conducted a multicenter, randomized, parallel-group, Phase II trial to evaluate the efficacy and safety of two doses of ofatumumab combined with fludarabine and cyclophosphamide (O-FC) in patients with previously untreated CLL. *Methods.* Patients with active CLL were randomized to ofatumumab 500 mg (Group A) or 1000 mg (Group B) Day 1, with fludarabine (F) 25 mg/m^2 and cyclophosphamide (C) 250 mg/m^2 Days 2-4, Course 1; Days 1-3, Courses 2-6; every 4 weeks for 6 courses. The first ofatumumab dose was 300 mg in both Groups. Dose reduction was allowed for FC only. Premedication for ofatumumab was paracetamol and antihistamine prior to each infusion and glucocorticoid prior to infusions 1 and 2. Growth factors and anti-infective prophylaxis were not mandated. The primary endpoint was complete response (CR; based on 1996 NCI-WG criteria) rate evaluated by an Independent Review Committee during treatment until 3 months after last dose. *Results.* All patients provided informed consent. A total of 61 patients were randomized (Group A, n=31; Group B, n=30). Pretreatment characteristics were as follows: median age 56 years (range, 38-73); Rai stage III-IV in 46%; bulky (>5 cm) lymph nodes in 16%; median serum β2-microglobulin level 4 mg/L (range, 1.8-11.5); median ALC 89 ×10^9/L (range, 3-307); 17p del in 13%; 11q del in 16%; unmutated IGHV in 41%. CR rate was 32% and 50% for Group A and B; ORR was 77% and 73%, respectively. Responses by baseline and treatment parameters are shown (Table). Short median follow-up (8 months) currently does not permit analyses of time-to-event endpoints. Ofatumumab C_{max} (426 *vs.* 201 mg/L) and C_{min} (60 *vs.* 20 mg/L) at 6th infusion were higher in Group B *vs.* Group A. Based on exploratory univariable regression analyses, lower β2-microglobulin (as continuous variable), completion of 6 courses of O-FC, and higher ofatumumab C_{min} prior to last infusion were significantly correlated with increased likelihood for response including CR, and decreased risk for progression. *Conclusions.* O-FC is highly active in previously untreated patients with CLL with CR rates up to 50%. β2-microglobulin level, number of O-FC courses completed, and C_{min} prior to last course correlated with outcomes with O-FC. Follow-up continues for time-to-event endpoints.

Table 1.

Parameters	N	% CR	% ORR
All patients	61	41	75
Age <65 yrs	50	44	76
≥65 yrs	11	27	73
ALC <30 × 10^9/L	11	64	82
≥30 × 10^9/L	50	36	74
Lymph nodes ≤5 cm	51	39	78
>5 cm	10	50	60
Rai stage 0–II	33	36	73
III–IV	28	46	79
β2-M <4 mg/L	32	53	84
≥4 mg/L	28	29	68
CD38$^+$ <30%	48	44	77
≥30%	12	33	67
IGHV mutated	28	46	75
IGHV unmutated	25	36	84
17p del	8	13	63
11q del	10	40	70
trisomy 12q	9	56	56
No FISH abnormalities	7	71	100
13q del (sole)	25	32	80
Number of O-FC courses received:			
6	39	56	92
4–5	9	33	78
1–3	13	0	23

0770

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0771

INHIBITION OF THE CXCR4/CXCL12 AXIS USING PLERIXAFOR TO IMPROVE THE RESPONSE TO THERAPY IN CHRONIC LYMPHOCYTIC LEUKAEMIA

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Background. Within tissues, chronic lymphocytic leukaemia (CLL) cells occupy a microenvironmental niche with a complex inter-dependence on secreted factors and stromal ligands. The chemokine receptor CXCR4 is expressed consistently at high levels by CLL cells. The CXCR4 ligand CXCL12 is recognised to promote cell migration, to direct cell interactions, and to enhance CLL cell survival. Inhibition of the CXCR4/CXCL12 axis has been proposed as a potential therapeutic measure in CLL. *Aims.* Plerixafor is a competitive inhibitor of the CXCR4/CXCL12 axis that is entering clinical practice for a number of clinical indications. We examined the *in vitro* effects of plerixafor to test its potential role as an adjunct to chemotherapy in the treatment of CLL. *Methods. Ex vivo* CLL cells were cultured *in vitro* in conditions that promoted sustained survival. Cells were tested in the presence or absence of chemokine, chemotherapeutic agents, or plerixafor. Flow cytometry was used to test surface-expression of CXCR4, relevant receptor proteins, and for apoptotic cell death. Binding of plerixafor to CXCR4 was tested using a flow cytometric blocking assay. Signalling responses were tested by immunoblotting analysis of CXCL12-responsive phospho-proteins. Functional evaluations included morphological and morphometric analyses, immunocytofluorescence of cytoskeletal proteins, and transwell filter-migration assays. Results. CXCL12 caused down-regulation of its receptor on CLL cells and induced rapid phosphorylation of p44/42 Mitogen-activated protein kinase (MAPK). The recognised actin polymerisation, polarisation and chemotactic responses were also observed. Additionally we were able to demonstrate relevant late biosynthetic responses to CXCL12 including up-regulation of the adhesion receptors L-selectin and β1 integrins. Plerixafor was found to bind to CXCR4 at low doses and to act as an inhibitor, blocking the CXCL12-induced phosphorylation of target proteins and preventing functional responses to CXCL12. We were able to show low levels of phosphorylation of MAPK induced by plerixafor in some CLL cases, but no functional agonist effects were observed. Chemotherapeutic agents from a range of classes including steroid, fludarabine, and less conventional agents such as Imatinib, all caused up-regulation of CXCR4 with a mean increase of 2.8-fold at 24 hours. All chemotherapeutic agents tested induced death of CLL cells, although sensitivity to the agents was found to vary between cases. The addition of CXCL12 to cultures consistently reduced the chemotherapy-induced cell death. CXCL12 was active before the formation of recognisable nurse-like cells suggesting direct pro-survival effects. Plerixafor alone did not induce death of CLL cells, but was able to prevent the pro-survival effects of CXCL12 in cells treated with chemotherapy. *Summary/Conclusions.* We have shown that the expression of CXCR4 is up-regulated in response to chemotherapeutic agents, and we suggest that this up-regulation may protect CLL cells from the effects of chemotherapy: by promoting the CXCL12-driven movement of CLL cells into the protective or proliferative environment; by causing phenotypic changes that favour their retention in a tissue environment; and by directly protecting from chemotherapy-induced death. We have demonstrated that plerixafor inhibits these potential chemo-protective effects, and suggest that the drug may have value as an adjunct to established or emerging chemotherapy regimes in CLL.

0772

PRELIMINARY RESULTS FROM A PHASE I DOSE ESCALATION STUDY TO DETERMINE THE MAXIMUM TOLERATED DOSE OF PLERIXAFOR IN COMBINATION WITH RITUXIMAB IN PATIENTS WITH RELAPSED CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Dose intensive rituximab in previously-treated patients with CLL has demonstrated modest activity. Preclinical data indicated that the CXCR4/SDF1 axis plays a key role in CLL cell homing and retention in tissue microenvironments, such as the bone marrow. Disruption of these interactions using the small-molecule CXCR4-antagonist, pler-

ixafor, could potentially mobilize CLL cells from the tissues to the blood, and thereby enhance their sensitivity to rituximab *in vivo*. To test this hypothesis, we initiated a phase 1 trial of plerixafor + rituximab in previously-treated patients with CLL. *Aims.* The primary objective was to determine the maximum tolerated dose (MTD) and safety of plerixafor when combined with rituximab. *Methods.* Rituximab-sensitive adult patients≤80 years old with WBC≤ 50×10^9/L, intermediate/high risk CLL and a diagnosis of active disease by NCI criteria were eligible to enroll. Patients were treated with 3x/week of rituximab, administered as a 100 mg flat dose on Day 1, and thereafter at 375mg/m^2 IV for 12 total doses. Plerixafor was administered SC, beginning with the 4th rituximab dose, 4 hours prior to rituximab in escalating doses of 0.08mg/kg, 0.16 mg/kg, 0.24 mg/kg and 0.32 mg/kg. Dose-limiting toxicities (DLT) were defined as any of the following, occurring from the first plerixafor dose (Day 8) through Day 29 and considered study drug-related: non-hematologic toxicity≥ grade 3, neutropenia≥ grade 3 which does not resolve to ≤grade 2 by week 4 of therapy in patients with normal ANC prior to therapy, hyperleukocytosis (WBC count>200×10^9/L) or tumor lysis syndrome requiring dialysis. MTD was defined as the highest dose at which no more than 1 of 6 patients experience a DLT. CD34$^+$ and CLL cells were enumerated in peripheral blood (PB) on Days 8 and 26 by flow cytometry; PB samples were obtained at baseline pre-plerixafor treatment and at 2, 4, 6, 10, and 24 hours post-plerixafor. *Results.* 14 patients (median 62 years; Rai Stage IV: 57%) have been enrolled, 3 patients each in the 0.08 and 0.24 mg/kg cohorts and 4 patients each in the 0.16 and 0.32 mg/kg cohorts (Table 1). No DLTs have been reported. Treatment-emergent, plerixafor-related adverse events (AEs) were seen in 6 patients and included diarrhea, vomiting, nausea, back pain, appetite loss and paraesthesia. All AEs were grade 1 in intensity except nausea that was grade 2 and back pain that was grade 3. Treatment-emergent serious AEs were seen in 2 patients (0.16 mg/kg dose; grade 2 EBV infection, grade 2 gastrointestinal reflux disease and grade 2 dyspnea); all unrelated to plerixafor. On Day 8, there was a median 3.4-fold increase in peripheral CLL cells (range: 1.2-11.7-fold), indicating CLL cell mobilization. On Day 26 fewer CLL cells were detected in the periphery with a median fold increase of 1.6 (range, 0.9-8.0). *Conclusions.* These preliminary data suggest that the combination of plerixafor plus rituximab in CLL patients appears to be well tolerated. Furthermore, plerixafor treatment can mobilize CLL cells to the blood, thereby justifying further investigation into its use as a potential sensitizing agent to enhance the activity of other agents utilized in the treatment of CLL.

Table 1. Patient characteristics and outcomes.

	0.08 mg/kg n=3	0.16 mg/kg n=4	0.24 mg/kg n=3	0.32 mg/kg n=4	Overall n=14
Median Age (Range)	69.0 (67-71)	53.0 (52-54)	60.0 (47-72)	65.5 (56-69)	62 (47-72)
Gender; male, n (%)	2 (67)	4 (100)	2 (67)	4 (100)	12 (86)
Ethnicity-Caucasian, n (%)	3 (100)	4 (100)	3 (100)	4 (100)	14 (100)
Rai Stage (at study entry), n (%)					
0	0	0	0	0	0
I	0	3 (75)	1 (33)	2 (50)	6 (43)
II	0	0	0	0	0
III	0	0	0	0	0
IV	3 (100)	1 (25)	2 (67)	2 (50)	8 (57)
Patients with Prior Rituximab Treatment, n (%)	3 (100)	4 (100)	3 (100)	3 (100)#	13 (100)#
Fold Increase in CLL cell mobilization on Day 8, Median (Range)*	5.00 (2.3- 6.9)	4.20 (2.4- 7.0)	3.80 (2.8- 11.7)	2.55 (1.2- 6.4)	3.4 (1.2-11.7)
Fold Increase in CLL cell mobilization on Day 26, Median (Range)*	2.40 (1.7- 5.4)	3.20 (1.3- 8.0)	1.00 (0.9- 1.1)	1.45 (1.1-3.2)	1.60 (0.9- 8.0)
Patients with Plerixafor-related Adverse Events, n (%)	1 (33)	1 (25)	1 (33)	3 (75)	6 (43)
Patients with Serious Adverse Events, n (%)	0	2 (50)	0	0	2 (14%)

*Fold Increase is calculated as Maximum Post-Dose value divided by the Pre-Dose value for that day
Data not available for 1 patient

0773

PERSISTENT PHENOTYPIC COMPLETE REMISSION OR MAINTENANCE TREATMENT PREDICT FOR LONG-LASTING RESPONSE DURATION AND LONGER OVERALL SURVIVAL IN CHRONIC LYMPHOCYTIC LEUKEMIA

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The treatment goal of chronic lymphocytic leukemia (B-CLL) is now the attainment of maximal disease control combining purine analogs with monoclonal antibodies. This approach have produced more complete molecular remissions and longer response duration (RD), often remaining only a minimal residual disease (MRD) detectable by flow cytometry. From 1998 to 2008, we treated in first line 126 B-CLL symptomatic patients (pts), median age 63 years, with six monthly courses of intravenous or oral fludarabine at conventional doses and then, after a median time of 31 days, with four weekly doses (375 mg/sqm) of rituximab (rtx). Fourteen pts had a low Rai stage, 109 an intermediate stage and 3 a high stage. We defined as high risk pts having at least two of these markers: unmutated IgVH, CD38>30%, ZAP-70>20%, intermediate/unfavorable cytogenetics (trisomy 12 or del11q or del17p). Fifty-one pts (40%) belonged to the high risk subset. For MRD flow cytometric study, the threshold was set at >1% CD19+CD5+CD79b- bone marrow (BM) CLL cells. Based on NCI criteria, 96/122 (78%) pts achieved a CR, 24/122 (20%) a partial remission (PR) and 2/122 (2%) no response or progression. Phenotypic CR (CD19+CD5+CD79b- BM cells <1%) was achieved in 71/122 (58%) pts. Ten pts underwent grade 3 (WHO) infective lung toxicity, 1 patient acute fatal B hepatitis and 2 pts progressed towards Richter's syndrome. Hematologic toxicity included mainly neutropenia (grade 3 and/or 4 in 60 pts) and thrombocytopenia (grade 3 and/or 4 in 8 pts). Fifty-four pts either in CR with B-CLL BM cells >1% (MRD$^+$, n=16 pts) or in CR MRD negative, but with B-CLL peripheral cells going up >1000/microl within 2 years after induction (n=22 pts) or in PR (n=16 pts), underwent consolidation and maintenance therapy with four monthly cycles of rtx at 375 mg/sqm followed by twelve monthly low doses of rtx (150 mg/sqm). The median follow-up duration was 53 months. All treated pts experienced a long progression-free survival from the end of induction treatment (40% at 9 years). Overall survival (OS) was 37% at 10 years from the start of treatment. Noteworthy, both persistently MRD negative (>1 year) pts (n=44) and pts undergoing consolidation and maintenance therapy (n=54) showed a longer RD *vs.* MRD$^+$ not consolidated pts (n=21) [97% *vs.* 76% *vs.* 10% at 4 years; P<0.00001, Figure]. Equally, global OS was shorter in MRD+ not consolidated pts in comparison with the other subsets (17% *vs.* 73% *vs.* 97% at 15 years; P=0.02, Figure). Noteworthy, within the high risk subset (n=51), both pts in persistent phenotypic CR (n=13) and consolidated pts (n=18) showed a longer RD (88% *vs.* 60% *vs.* 0% at 2.6 years, P=0.007) *vs.* MRD+ not consolidated pts (n=14). In our experience, both persistent MRD negativity and rituximab maintenance therapy improve RD and OS in B-CLL, also within the high risk subset.

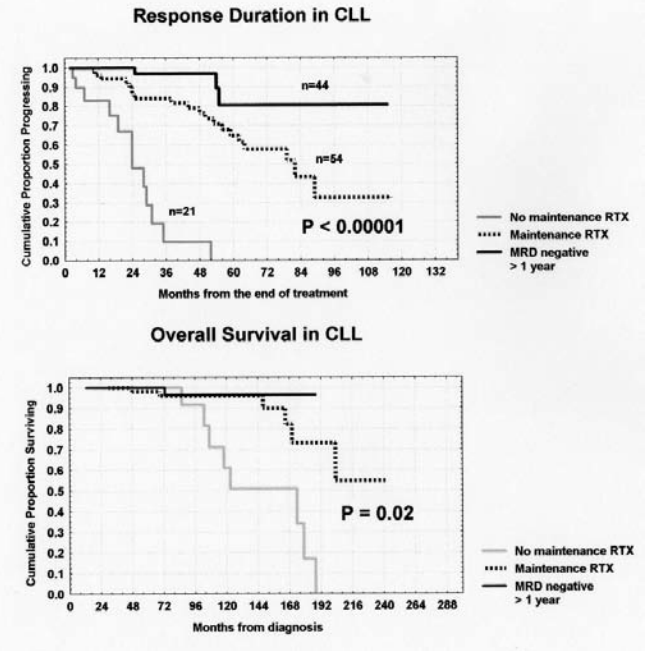

Figure 1. Response duration and overall survival in CLL

0774

BENDAMUSTINE (B) IN COMBINATION WITH RITUXIMAB (R) FOR PATIENTS WITH RELAPSED/RESISTANT CHRONIC LYMPHOCYTIC LEUKEMIA (CLL): AN ITALIAN MULTICENTRE RETROSPECTIVE STUDY

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Background. Current second-line therapies for CLL produce unsatisfactory clinical *Results.* Bendamustine has shown a considerable degree of efficacy in patients with lymphoid malignancies. There is evidence that the *in vitro* cytotoxic effect of Bendamustine may be enhanced by combination with Rituximab. Encouraging results have been reported with this association (R-B) for the treatment of NHL but data on CLL are scanty. Aim of this study was to retrospectively assess the efficacy and toxicity of the R-B association in a multicentre cohort of relapsed/refractory CLL patients. *ethods.* 109 CLL patients relapsed following, or refractory to, first line therapy including Rituximab and/or fludarabine were treated with R-B in 24 Italian centres. Data on diagnosis and relapse, treatment, toxicity, and outcome were analysed. Results. Patients M/F ratio was 2/1, all had Binet stage C or B and active disease; the median age was 66 years (range 39-85), 39% showed elevated LDH value (median[3DOTS]), 18% had B-symptoms, 43% were relapsed and 57% were resistant after a median of 3 lines of previous therapy (range 1-8). 22 patients received bendamustine alone and 88 patients were given R-B (median B dosage: 100 mg/m^2/d, range 90-130 mg/m^2/d). The ORR was 69.6% (CR=28.6%; PR 41%) and a 26% CR was achieved within the 4th chemotherapy cycle. The ORR was significantly higher in patients treated with R-B (P=0.014) and in those responsive to the latest chemotherapy course (P=0.04). After a median follow-up of 7.9 months (range 1-148), 35% patients had progressed and 31% had died. The median PFS was 16 months and the median Duration-of-Response was 13 months. Patients who achieved a CR had a significantly longer DOR than those in PR (not reached *vs.* 8 months). Median OS was 16.8 months; disease status before Bendamustine administration (relapse *vs.* resistant P<0.0001) and the achievement of a CR (vs PR P=0.006) significantly influenced the OS. In a multivariate Cox model, resistant disease status at start of Bendamustine treatment (HR 3.2, 95% CI 1.4-7.3, P=0.006) and unresponsiveness to R-B (HR 4.5, 95% CI 2.5-10.5, P<0.0001) remained independent prognostic factors for OS. A total of 460 cycles were administered, (median 4 cycles/patient). Grade 3/4/5 CTC toxicity occurred in 56 of the 460 treatment cycles: 5 were infections (4.5%) [herpes-zoster (n=2), herpes encephalitis (n=1), bacterial pneumonia (n=1) and pulmonary aspergillosis (n=1)], and 52 were haematological toxicities. Overall, grade 3/4 anemia was recorded in 16% of patients, neutropenia in 17% and thrombocytopenia in 16%. Three out of 34 (9%) deaths (treatment-related mortality amounted to 2.7%) were due to infections [herpes encephalitis (1), bacterial pneumonia (1) and pulmonary aspergillosis (1)]. Conclusion. Our data demonstrate that patients treated with R-B association can achieve a remarkable ORR and CR rate in relapsed/refractory CLL patients. Responses were obtained promptly and were burdened by limited toxicity, showing that this chemotherapy-regimen should be considered an effective therapeutic option for this group of patients. These findings suggest that further studies aimed to compare the efficacy of R-B with fludarabine, cyclophosfamide and Rituximab should be strongly encouraged.

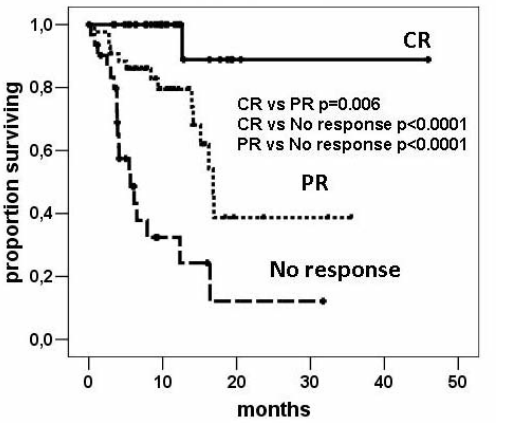

Figure 1. Overall survival.

0775

FLOW CYTOMETRIC MRD NEGATIVITY AT DAY 365 AFTER ALLOGENEIC STEM CELL TRANSPLANTATION (SCT) PREDICTS BETTER THREE-YEAR PROGRESSION-FREE SURVIVAL (PFS) IN PATIENTS WITH ADVANCED B-CLL

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Background. In a completed study (NCT00337519), patients with advanced B-CLL received allogeneic SCT after cytoreductive treatment with alemtuzumab followed by a wash-out period for the antibody and conditioning with fludarabine/busulfan. Aim of the present investigation was to correlate flow cytometric MRD levels in the peripheral blood at different time points after transplantation with patient outcome. *Methods.* In 58 CLL patients 900 flow cytometric MRD investigations (at least 4 measurements/patient: at day 30, between d31-100, d101-180, and d181-365 after SCT) were performed measuring the following CLL phenotype: CD19posCD5posCD20dimCD79bneg. Therefore, a 4-color-approach (until 2006) or an 8-color-approach then in combination with T- and NK cell antigens was performed. A patient was defined as MRD negative if less than 0.05% CLL cells were detectable or as relapse if more than 0.05% CLL cells were redetectable in at least two successive investigations. For assessment of PFS, clinical progress was defined according to the NCI criteria. *Results.* The median follow up time after SCT was 536 days (range: 44d-1758d). Considering all 58 transplanted patients the probability of three-year overall survival (OS) including the 95% confidence interval was 64±19% (1-year: 83±10%) and of three-year PFS 45±17% (1-year: 74±12%). In the majority of cases flow cytometric MRD negativity was achieved within the first year post SCT with a cumulative incidence of 36±13% at day 100 and of 73±12% at one year, respectively. Only two additional patients became MRD negative within the second year post SCT. Patients who achieved MRD negativity until day 365 showed a significantly better 3-year OS compared to the MRD positive group (90±15% *vs.* 56±49%; P=0.009). Remarkably, the 3-year PFS of patients achieving flow cytometric MRD negativity until day 365 was also significantly better than in the MRD positive cases (75±20% *vs.* 0%; P=0.002). Of note, early flow cytometric MRD negativity until day 100 was not informative concerning one-year OS or PFS (88±13% *vs.* 79%±15% and 77±18% *vs.* 72±18%). The flow cytometric MRD status was one trigger to give DLI. Interestingly, this kind of immunomodulation resulted in flow cytometric MRD negativity in all eight patients after a median of 130 days. The probability of relapse in the investigated patient cohort was 15±10% after 1 year and 31%±14% after 3 years. Thus, 9/14 patients showed a clinical relapse in parallel with flow cytometric

MRD positivity. Two patients featured an isolated flow relapse with MRD at two successive investigations. Three patients showed a mere nodal relapse, all occurring within the first year post SCT. *Conclusions.* In summary, the present flow cytometric MRD study in B-CLL patients elucidates the dynamics of remission induction and relapse in the first year post SCT. In the majority of patients MRD is eradicated between day +100 and day +365 which is the time interval when chronic GvHD occurs in most cases. Therefore, close monitoring of MRD status in the first year after SCT is necessary. Once patients are flow cytometrically MRD negative at day 365, they seem to have a high probability of long term survival.

0776

MONOCLONAL B-CELL LYMPHOCYTOSIS (MBL) WITH NORMAL WHOLE LYMPHOCYTE COUNTS IS ASSOCIATED WITH REDUCED NUMBERS OF NORMAL CIRCULATING IMMATURE AND MATURE B-LYMPHOCYTES

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Background. "Monoclonal B-cell lymphocytosis"(MBL) is the international consensus term proposed to indicate the presence of $<5\times10^9$/L circulating monoclonal B cells -with or without increased B-cell counts- in otherwise healthy subjects. Normal B- and T- lymphocytes have been found to be decreased in patients with chronic lymphocytic leukemia (CLL) but until now there is no information about this matter in healthy subjects with MBL. *Aims.* To investigate the distribution -in terms of circulating absolute numbers- of B-, T-, and NK-cell subsets in MBL cases. *Methods.* Using a high-sensitive flow cytometry approach, based on 8-color staining panels and the systematic screening for $>5\times10^6$ total PB leucocytes, we immunophenotyped a total of 95 peripheral blood (PB) samples of healthy subjects with MBL (55 f / 40 m, aged 70±11 years) showing normal lymphocyte counts ($2.2\pm0.6\times10^9$/L). As a control group we analyzed 653 age- and sex-matched healthy subjects with no evidence of a clonal disease. *Results.* Absolute numbers of normal circulating B-cells and all the different maturation B-cell subsets (immature, naïve, memory B-lymphocytes and plasmablasts) were significantly reduced in MBL cases *vs.* controls. In an additional applied aged-matched comparison, immature B cells and $CD4^+/CD8^+$ T cells were also decreased ($P<0.05$) in MBL *vs.* controls. Furthermore, a more progressive decrease in the numbers of circulating B-cell subsets such as immature, naïve, IgM negative memory B-lymphocytes and plasmablasts (but neither in T- nor in NK-cells) was observed, with statistically significant differences at a cut-off point of 1% monoclonal B-lymphocytes in PB. Interestingly, lower absolute numbers of both CD5 neg. immature and naïve B-cells were detected in those MBL cases displaying two B-cell clones in comparison to single clone MBL-subjects, particularly in those biclonal MBL cases carrying one CLL-like clone plus one CD5 negative non-CLL clone ($P<0.05$). Moreover, a significantly higher amount of clonal B-cells *vs.* both monoclonal MBL and CLL-like+CLL-like biclonal MBL were observed in biclonal cases carrying one CLL-like clone plus CD5 negative non-CLL clone (monoclonal CLL-like MBL: $20\pm145\times10^6$ clonal cells/L; CLL-like+CLL-like biclonal MBL: $5\pm10\times10^6$ clonal cells/L and CLL-like plus CD5 negative non-CLL biclonal MBL: $463\pm541\times10^6$ clonal cells/L; $P<0.05$). *Conclusions.* Overall, our results show that MBL cases have reduced absolute numbers of both total normal B-cells and their different B-cell subsets identified in PB, in comparison to controls. This decrease seems to be dependent on the amount of circulating clonal B cells, suggesting a suppressive effect of the MBL-clone on normal B-cell lymphopoiesis. Whether this suppression may be derived from a competitive situation concerning the B-lymphocyte production in the stem cell niche requires further investigations.

0777

BENDAMUSTINE AND ALEMTUZUMAB (BEN CAM) COMBINATION IN RELAPSED AND REFRACTORY CHRONIC LYMPHOCYTIC LEUKAEMIA (CLL): PHASE I SAFETY PROFILE RESULTS OF THE ITALIAN TRIAL

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Single-agent alemtuzumab has been shown to be effective when used either as first-line therapy, or in relapsed/refractory CLL. Bendamustine is a bifunctional alkylating agent with considerable activity both in monotherapy or in combination with other cytostatic agents in relapsed /refractory CLL and indolent lymphomas. Both agents exhibit an individual, unique mechanism of action. Therefore, a synergistic or additive effect might be expected when they are used in combination. Ben Cam study is a multicentric Italian, single arm, open label, dose escalation study designed to determine the best tolerable treatment schedule and efficacy of the combination of bendamustine and alemtuzumab in patients with refractory/relapsed CLL. In the first part of the study, the tolerability of a stepwise dose-escalation schedule of bendamustine along with an increasing dose of alemtuzumab has been determined. This schedule explored the Dose-Limiting Toxicities (DLT) and the Maximum Tolerated Dose (MTD) of this treatment. Bendamustine was given at a starting dose of 50 mg/m^2 for 2 consecutive days along with alemtuzumab 20 mg sc on days 1 to 3. If MTD was not reached within the first cohort of 3 patients the second cohort of 3 patients received the same dose of bendamustine with an increased dose of alemtuzumab (30 mg). If the MTD was not reached after completion of Cohort 2 the third cohort of 3 patients received bendamustine 70 mg/m^2 along with alemtuzumab 30 mg sc. A further dose escalation is not foreseen. If one of 3 patients at each dose level experienced DLT three additional patients were added (max 6). If 5 of the 6 subjects were able to complete the first 2 cycles without experiencing DLT, this level was considered tolerable and dose-escalation continued initiating the following cohort. This combination was repeated on day 29 for up to 4 cycles. Twelve patients have been enrolled in the Phase I of this trial: 3 patients in each of the first 2 cohorts, 6 in the third cohort. The median age was 63.7 years (range, 52-77), seven male (58%), 33% had Binet stage C, median number of prior regimens was 2 (range, 1-4). Seven patients received monoclonal antibodies as previous treatment: rituximab in 3 cases and alemtuzumab in 4 cases. Unmutated IGVH genes were detected in 8/12 (66%) patients. Grade III-IV neutropenia episodes were observed in 58%, 19%, 24% of courses in the first, second and third cohort respectively. Grade III-IV thrombocytopenia episodes were detected in 0%, 18%, 0% of cycles in each cohort respectively. Grade III-IV anemia episodes were recorded in 8.3%, 0%, 0% in each cohort respectively. One major infection sustained by Pneumococci (pneumonia) was observed. CMV reactivation occurred in 4 patients: no CMV disease was recorded. Extra-hematological toxicity was mild. Therefore, bendamustine 70 mg/m^2 along with alemtuzumab 30 mg sc resulted as a tolerable treatment. In the second part of the study, patients will receive this dose level to provide data on the anti-tumor activity of the combination.

0778

SUPPORTING RITUXIMAB THERAPY IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. The application of the combination of rituximab with fludarabine-containing chemotherapy regimen in recent years significantly improved the results of overall and disease-free survival of patients with chronic lymphocytic leukemia (CLL). However, to avoid the relapses of a certain group of patients, unfortunately, is not possible, because it is an open question about the possibility of complete eradication of malignant clone in this neoplasia. Therefore, the main task of this modern stage is to develop a strategy lengthening the period till disease progression. *Aim.* The aim of the presented work was to study efficiency of supporting rituximab therapy after induction chemotherapy or immuno-chemotherapy in CLL patients. *Methods.* The study

included 193 patients in remission. The age of patients ranges from 38 to 74 years (a median - 59 years). Complete remission (PR) was observed in 110 (57%) patients, partial remission (CR) in 83 (43%). Remission of the disease was obtained as a result of induction chemotherapy RFC program (rituximab, fludarabine, cyclophosphamide) in 107 patients or FC (fludarabine, cyclophosphamide) in 86 patients. Program RFC included: rituximab 375 mg/m^2 i/v in 1 day, fludarabine 25 mg/m^2 i/v 2-4 days and cyclophosphamide 300 mg/m^2 i/v 2-4 days. Program FC included fludarabine 25 mg/m^2 i/v 1-3 days and cyclophosphamide 300 mg/m^2 i/v 1-3 days. After achieving the overall efficiency, the patients were randomly assigned to either observation (133 patients) or supporting rituximab therapy in the form of 4 weekly injections (375 mg/m^2) every 6 months withing 2 years (60 patients). *Results.* A result of the work showed that patients with CLL who got RFC regimen with subsequent supporting rituximab therapy, had the frequency of relapses and deaths significantly lower compared with patients who were observed without supporting rituximab therapy (P=0.001, P=0,015, respectively). Analyzing these indicators in patients treated with FC, followed by supporting rituximab therapy, we see advantage of maintenance therapy in relation to the observation group (P=0.0001, P=0.002, respectively). Comparative analysis of progression free survival (PFS) CLL patients, who received various regimens, revealed a significant difference. Thus, in patients who completed the program, followed by RFC and supporting rituximab therapy, the median PFS has not been achieved, while in patients without supporting rituximab therapy, it was 42 months (P=0.009). The related indicators of patients receiving the combination of FC with further supporting rituximab therapy (P=0.004) differed significantly, their median PFS was not achieved in contrast to patients of the monitoring group, whose PFS is 24 months (P=0.001). During the period of supporting rituximab therapy, the signs of hematological toxicity did not exceed the I stage. The number of infectious complications tended to be equal in observation group and in the group of patients receiving supporting rituximab therapy and was 7%. Summary. Thus, the results of the study showed the role of supporting rituximab therapy in CLL treatment effectiveness. With the help of supporting rituximab therapy it is possible to maintain a more-expendable remission and overall satisfactory condition of the patients without signs of disease progression, and thereby improve long-term results of tumor therapy process.

0779

ACADESINE FOR PATIENTS WITH RELAPSED/REFRACTORY CHRONIC LYMPHOCYTIC LEUKEMIA: A MULTICENTRE PHASE I/II STUDY

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Acadesine induces cell death in B-cell chronic lymphocytic leukemia (CLL) cells in a dose-dependent manner. Acadesine enters B-cells where it is phosphorylated to ZMP, which induces apoptosis independently of ATM or p53. It is active *in vitro* against CLL cells from patients who have not responded to prior treatment with fludarabine and/or chlorambucil. A phase I/II open-labeled clinical study was designed to determine the safety and tolerability of acadesine given intravenously as a 4-hour infusion to patients with CLL. Part I is the dose escalation part of the study where patients receive a single dose of acadesine on Day 1 and are followed up to Day 22. In Part II, patients will receive up to 5 doses of acadesine at the maximum tolerated dose (MTD) identified in Part I over a period of up to 20 days. Patient population includes CLL patients with relapsed/refractory disease who have received one or more prior lines of treatment including either a fludarabine or an alkylator-based regimen. A patient is defined as having refractory disease if they fail to achieve less than a partial response (PR) according to the NCI working group guidelines, or relapse within the first 6 months after treatment after achieving at least partial response. Primary endpoints of the study evaluate the safety and tolerability of acadesine. Secondary endpoints evaluate the pharmacokinetics of acadesine and ZMP, and B and T-cell counts in peripheral blood as efficacy biomarkers. Eighteen patients have been included to date in Part I at doses of 50, 83.5, 139.5, 210 or 315 mg/kg. Pharmacokinetic data showed acadesine is rapidly converted into ZMP inside blood cells. The Cmax levels for ZMP in whole blood obtained at 315 mg/kg were similar to the ones obtained at the previous dose (210 mg/kg), suggesting that the saturation plateau was reached, which will be confirmed based on PK modeling. In 5 patients treated with acadesine at 210 mg/kg and 315 mg/kg a decrease in absolute B cell count was observed, ranging from 6% to 54% with respect to the B cell count prior to acadesine administration. Reversible asymptomatic hyperuricaemia was observed in four patients in cohorts 1 to 3, probably due the metabolism of acadesine to ZMP and uric acid. Prophylactic allopurinol was used in cohorts 4 and 5 and it has significantly reduced the incidence of hyperuricaemia. Acadesine 315 mg/kg was the dose limiting toxicity (DLT) dose with 2 of 3 patients having DLTs-Tumour Lysis Syndrome (TLS) and clinically significant acute renal failure (CTCAE V3.0 Grade 3-chronic dialysis not indicated). Additional safety, pharmacokinetics and efficacy data will be presented at the meeting. In conclusion, a MTD was found and multiple dose acadesine administration is currently planned in part II of this ongoing study.

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DOSE DENSE HIGH DOSE METHYLPREDNISOLONE (HDMP) AND RITUXIMAB (RTX) ARE EFFECTIVE IN RELAPSED OR REFRACTORY HIGH RISK CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

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Background. Management of high risk CLL remains a considerable challenge. Despite progress using chemoimmunotherapy, many patients would not tolerate the treatment due to toxicity. There is a low response rate in cases with 17p del/p53 mutation. Alemtuzumab has limited activity in patients with bulky lymphadenopathy. Aim of the study. was to evaluate the efficacy and safety of dose dense HDMP and Rtx combination in a prospective study. *Methods.* Patients were included if they had a relapsed or progressive disease after at least one line of chemotherapy with adverse cytogenetics (17p del, p53 mut, 11q del and/or trisomy 12) and / or progressed within 12 months of fludarabine treatment. All patients provided informed consent. Patients were required to have an indication for treatment based on the NCIWG-96 criteria. HDMP was administered at 1 g/m^2 intravenously daily for five consecutive days of each treatment course. Rtx was administered at a dose of 375 mg/m^2 on day 1 and 500 mg/m^2 on day 5 of the first course, on days one and five of the second course, and on day one of courses three to six. Courses were repeated every 21 day for a total of 6. Response was evaluated according NCIWG-96 criteria. All patients underwent a bone marrow biopsy and a CT scan for residual disease. *Results.* 29 patients with CLL were enrolled. Median age was 59 years (range 45-76), 22 (76%) patients had Rai III-IV stage, 17 (59%) had bulky (> 5 cm) lymphadenopathy. 25 (86%) patients had unmutated IgVH, 13 (45%) had 17p del and/or p53 mutation, 11 (38%) had 11q del, and one (3%) patient had trisomy 12, 10 (34%) patients progressed within 6 months of fludarabine treatment. Overall response rate (ORR) in 26 evaluable patients was 69%, one patient (4%) had complete response, 17 (65%) patients achieved partial response (PR), and stable disease was confirmed in 8 (31%) patients. There were no significant differences in ORR in different risk groups: 90% in patients with 17p del/p53 mutation, 64% in patients with 11q del, 70% in fludarabine refractory patients and 62% in patients with bulky lymphadenopathy (P=0.34). 4 patients with PR underwent allogeneic bone marrow transplantation. After the median follow-up of 19 months, the median progression free survival was 12 months (range 10-14) and median overall survival was not reached. The most common toxicity was hyperglycemia not requiring intervention in most cases. 12 cases of III-IV° neutropenia and 2 cases of febrile neutropenia were observed. III° infections were noted in 4 cases. There were 3 early deaths during treatment: one sudden death of unknown reason, one of sepsis, and one death of gastrointestinal bleeding due to preexisting thrombocytopenia. *Conclusions.* The dose dense treatment with HDMP and Rtx is an effective therapy with favorable safety profile in high risk CLL patients, including those with 17p del/p53 mutation. A follow-up study is planned. (ClinicalTrials.gov identifier: NCT005 58181; in part supported by EEA and Norway grant No. 2004-LT0040-IP-1EEE.)

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RITUXIMAB IN COMBINATION WITH HIGH-DOSE DEXAMETHASONE: AN EFFECTIVE TREATMENT OPTION FOR PATIENTS WITH RELAPSED/REFRACTORY CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Patients with refractory CLL have poor outcome despite currently used salvage treatment. Regimens based on high-dose corticosteroids seem to offer a promising treatment option in this scenario. High-dose methylprenisolone combined with rituximab (R-HDMP) demonstrated significant activity in relapsed/refractory CLL but serious infectious complications occurred in a substantial proportion of patients. Pilot data have shown that combination of dexamethasone and rituximab (R-Dex) may provide comparable results with less toxicity. *Aims.* and *Methods.* We performed a retrospective analysis of the efficacy and safety of R-Dex in patients (pts) with CLL treated at two tertiary centers between April 2006 and February 2010. Patients received two versions of R-Dex regimen: the dose of rituximab was either 500 mg/m^2 on day 1, 8, 15, 22 (375 mg/m^2 in 1st cycle), repeated every 4 weeks (n=25) or 500 mg/m^2 on day 1 (375 mg/m^2 in 1st cycle) repeated every 3 weeks (n=16). The dose of dexamethasone was identical in both regimens: 320 mg per cycle (40 mg on day 1-4 and 10-13 or 15-18). *Results.* R-Dex was administered to 41 patients (19 males) with median age of 68 years (range, 44-81) indicated for treatment according to NCI-WG criteria. Autoimmune hemolytic anemia or thrombocytopenia was the only indication for the treatment in 7 patients. Rai stage III/IV was present in 37/41 pts. IgVH genes were unmutated in 24/29 pts with available *Results.* Cytogenetic aberrations detected by FISH (n=33) revealed del 17p in 7 patients; del 11q in 11 patients; del 13q in 15 patients and trisomy 12 in 5 patients. Median number of previous therapies was 2 (0-8); 29/41 pts were previously treated with fludarabine-based regimens. The effect of R-Dex in evaluable patients without hemolysis (n=32) was: overall response rate (ORR), n= 21 (62%), complete remission (CR), n=6 (18%), partial remission (PR), n=15 (44%), stable disease (SD), n=4 (12%) and progressive disease (PD), n=5 (15%). All patients treated with R-Dex for autoimmune cytopenia achieved complete resolution of hemolysis. Grade III or IV toxicity included infections in 13 patients (32%), steroid diabetes in 6 patients (15%) and rituximab infusion-related side effects in 3 patients (7%). At the time of analysis (February 2010), median progression free survival (PFS) was 9 months; median overall survival has not been reached. There was no difference in ORR, PFS or OS between the two versions of R-Dex regimen. *Conclusions.* This pilot study shows that R-Dex is a feasible and effective treatment for relapsed/refractory CLL. In particular, R-Dex appears to be highly effective in CLL with autoimmune cytopenias. However, infectious toxicity remains a serious issue. In addition, long-term disease control can be expected in minority of patients only. Interestingly, higher dose of rituximab per cycle did not result in improved efficacy.

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0782

HISTONE DEACETYLASE INHIBITION IN CLL WITH TP53 DELETION/ MUTATION

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Introduction. Histone deacetylase inhibitors (HDACi) exhibit promising anti-cancer activity (e.g. tumor cell growth and survival inhibition and low toxicity in healthy cells), but their mechanism of action is not fully understood. There exists a discourse as to whether p53 plays a major role in HDACi-mediated cell death and few studies have examined this question in CLL. In this study, we examine HDAC-induced cell death with regards to TP53 status of primary CLL samples. *Materials and Methods.* An initial screen was performed on CLL cells from a genetically diverse cohort to test the efficacy of eight small molecule therapeutic agents with respect to p53 status in CLL primary samples. After initial positive results of the HDACi Trichostatin A (TSA), this compound was tested on three CLL cell lines (Mec1, Mec2, and EHEB) with 24h, 48h, and 72h incubation times and 23 primary CLL cell samples (8 of which with 17p deletion) using a chemiluminescence-based cell viability assay (CellTiterGlo, Promega) with 24 h and 48 h incubation times. These results were confirmed using FACS. HDACi with purportedly similar mechanisms, SAHA (Vorinostat), LBH589 (Panobinostat) and CHAHA, were tested on a subgroup. Results. Significant cell death was observed in all cell lines tested after 24 h incubation with 100 nM TSA. Near absolute cell death was observed after 48 h incubation with 1 μM TSA. Interestingly, Mec1 and Mec2 (both of which have TP53 mutations) exhibited a higher sensitivity to TSA than EHEB (wild-type TP53). Primary CLL cell samples exhibited sensitivity to 100 nM and 10 nM TSA after 24 h and 48 h, respectively. Similar to the cell lines, near absolute cell death was observed after 48 h with 100 nM TSA. Cases with 17p deletion (6/8 of which also had TP53 mutations) showed a distinctly (>two-fold) higher response to TSA than those without (Figure). SAHA and CHAHA were observed to induce less cell death in primary CLL cell samples than TSA at equimolar concentrations: sensitivity was observed after 48 h with 1 μM and 10 μM SAHA and CHAHA, respectively. Unlike TSA, neither SAHA nor CHAHA induced cell death more prominently in cells with 17p deletion. Conclusions. SAHA and CHAHA induced cell death in primary CLL cells irrespective of 17p status suggesting a p53-independent mechanism; however, TSA exhibited a more prominent effect in cell lines with TP53 mutation and primary cells with 17p deletion suggests a potential synthetic lethal interaction with mutant p53. The high sensitivity of primary CLL cells to HDACi at minute concentrations and their activity regardless of 17p-status are promising.

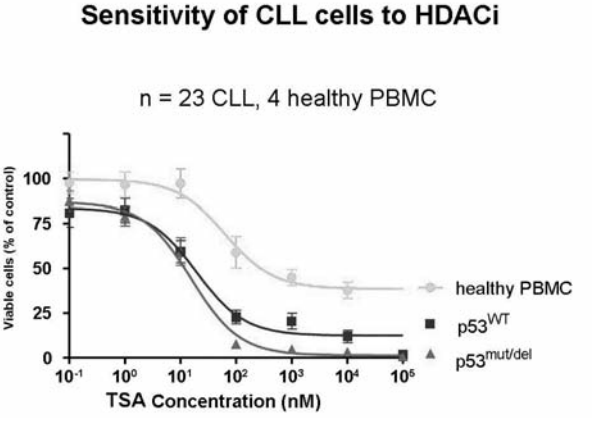

Figure 1. Effect of HDACi in CLL based on p53 status.

0783

A TRIPARAMETRIC QPCR SCORE COMPOSED OF ZAP70, LPL AND MICRORNA-29C AS A NEW PROGNOSTIC TOOL FOR CHRONIC LYMPHOCYTIC LEUKEMIA RISK STRATIFICATION AT THE TIME OF DIAGNOSIS

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Background. this last decade, several markers have been proposed to predict the outcome of chronic lymphocytic leukemia (CLL) patients. However, several discordances exist between prognostic factors indicating that no factor is totally perfect. The use of only one factor could thus lead to the misclassification of the patient. *Aim.* the present study aimed to investigate the prognostic power of new RNA-based prognostic markers and to assess the prognostic power of a quantitative PCR (qPCR) score composed of the most powerful factors in order to reduce patient misclassification. *Methods.* ZAP70, LPL, CLLU1, microRNA-29c and microRNA-223 were measured by real time PCR in a cohort of 170 patients (Binet stage A=124, B=31 and C=15) with a median follow-up of 64 months (range 3-330). For each patient, cells were obtained at diagnosis and RNA was extracted from highly purified CD19 cells. Each factor was first investigated individually for treatment-free survival (TFS) and overall survival (OS) by Kaplan Meier curves (compared with a log-rank test) and univariate Cox regression. The best markers were

included in a qPCR score which was thereafter compared to each individual factor but also to other classical prognostic markers (IgVH mutational status, Binet stage, CD38, sCD23, beta2-microglubulin, lymphocyte doubling time, cytogenetic abnormalities). *Results.* statistical analysis showed that all 5 RNA-based markers can significantly predict TFS but only ZAP70, LPL and microRNA-29c could significantly predict OS. These 3 markers were thus included in a new and simple qPCR score (where each poor prognosis marker corresponds to an increase of 1 unit) dividing patients in 3 groups (0/3, 1-2/3 and 3/3). This score significantly predicts TFS (P<0.0001) and OS (P=0.0001). Median TFS were >210, 61 and 24 months and median OS were >330, 242 and 137 months, respectively. TFS results were also confirmed in Binet stage A patients (P<0.0001; median TFS were >210, 88 and 34 months, respectively for group 0/3, 1-2/3 and 3/3) but OS analysis only exhibits a trend (P=0.0443) due to the limited number of event in stage A patients (n=8). When compared to other classical factors, this score displays the highest univariate Cox Hazard Ratio (TFS: HR=9.45 and OS: HR=13.88). Finally, this new prognostic tool allows the identification of patient groups with a higher median TFS and OS in poor prognostic subgroup and inversely. *Conclusions.* using a unique and standardized technique, we developed here a simple qPCR score to assess CLL patient prognosis in terms of TFS and OS. This score, able to classify patient in 3 different prognostic groups, seems to be up to now one of the most powerful markers for CLL risk stratification at the time of diagnosis.

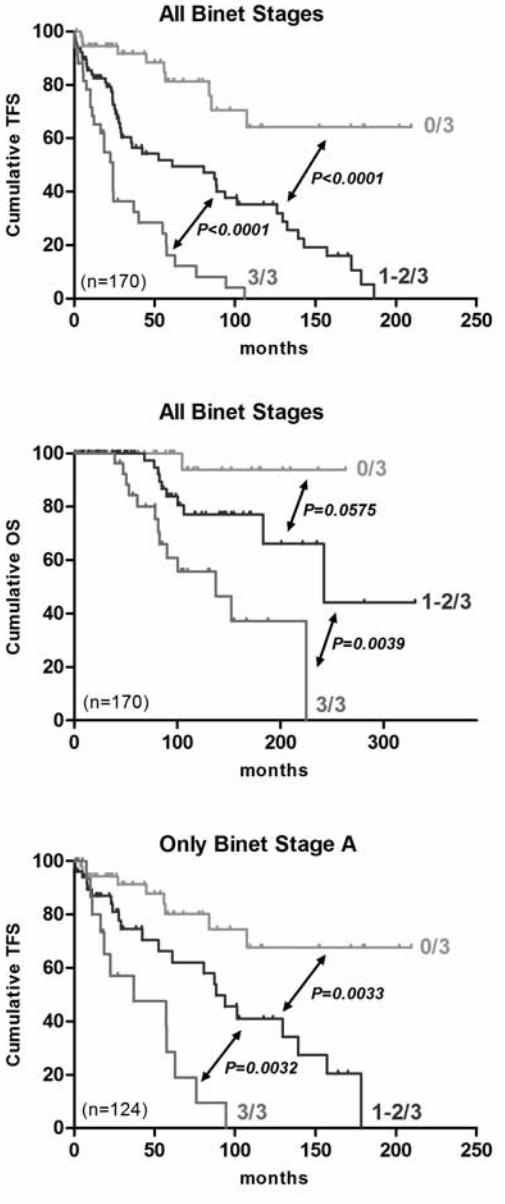

Figure 1. Prognostic value of the qPCR score.

0784

T-LARGE GRANULAR LYMPHOCYTOSIS (T-LGL) IN PATIENTS WITH CHRONIC NEUTROPENIA: EPIDEMIOLOGICAL CHARACTERISTICS, CLINICAL COURSE AND RELATION WITH CLONAL DISEASE

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Background. The disorders of T-LGL constitute a broad group of reactive or clonal conditions requiring further research attention. *Aim.* We studied patients presenting with chronic neutropenia at the Hematology Outpatient Clinic of the University of Athens and analysed the prevalence of T-LGL and clonality, as well as patients' clinical characteristics and course. *Methods.* Between 2005-2008 we prospectively studied patients with chronic neutropenia (<1.8×10^9/L, >6 months). The tests performed included clinical examination, complete blood count (CBC), blood smear examination, full biochemical profile, EBV and CMV antibodies, autoantibodies and blood immunophenotypic study with 9 monoclonal antibodies. Those with immunophenotypic evidence of T- LGL underwent a T-cell gene rearrengement (TCR αβ and γδ). If clonality was proved, patients underwent a thoracic and abdominal CT scan, as well as a bone marrow examination (BME). Patient's follow up was repeated every 3-6 months. *Results.* 224 patients were included in this study. The median duration of neutropenia until presentation was 4 years. Female to male ratio was 4:1with a median age of 55 years. Of the187 patients who underwent an immunophenotypic analysis, 54 were found to have T-LGL lymphocytosis. Female to male ratio in T-LGL group was 2:1, with a median age of 60 years (Table 1). No serious infections were accounted in the T-LGL group; 22/54 (41%) experienced recurrent apthous stomatitis, urinary infections, chronic prostatitis and recurrent lung infections as well as mild asthma and chronic obstructory pulmonary disease exacerbations. In 12/54 allergic conditions were seen. Only 4% presented a concurrent autoimmune disease. 24 patients (44%) had a thyroid disorder; commonly Hashimoto's thyroiditis. Ten patients presented with anemia (haemoglobin 9.2-11.4 g/dL), while 8 had a mild thrombocytopenia (platelets 92-130×10^9/L). Median number of leucocytes was 3.8×10^9/L, with a median neutrophil count 1.5×10^9/L (0.5-2.7×10^9/L). Median lymphocyte count was 1.6 ×10^9/L (0.7-4.3×10^9/L). In 41/54 patients of the T-LGL group a TCR study was performed and 26 were found positive. The severity and rate of infections, the prevalence of underlying conditions, presence of cytopenias, and thyroid disorders in this clonal T-LGL group did not differ from the rest of the patients. A bone marrow trephine examination was performed in 17 patients with clonal T-LGL. In 3 cases T-LGL leukaemia was confirmed histologically and with positive TCR rearrangement in the bone marrow. 11 BME described a scant interstitial and scattered (diffuse) lymphocytic infiltration, as well as reactive bone marrow disorders. It must be noted that in 7 of those patients, TCR of the bone marrow was positive. None of our patients has required specific treatment during their follow up, except from one who receives erythropoietin.

Table 1. Neutropenia and T-LGL patients' characteristics.

Characteristic	# (%)
Median time of duration of neutropenia : 5 years (1-20)	
Male	17/54(31)
Age at diagnosis <50 years	11(20)
Thyroid disease Collagen-tissue disease Malignancies	24 (44) 4 (7) 6 (12)
Infections: Reccurent Apthous ulcers	16(29) 6 (11)
Allergic conditions	12 (24)
Anaemia :Hb<13.5 g/dl, men Hb<12.0 g/dl, women	4 (24) 6 (16)
Thrombocytopenia (<150x10^9/l)	8(14)
Lymphocytosis >4.0x10^9/l	1 (2)
Lymphocytopenia <1.5 x10^9/l	19 (35)
ANA(>1:160)	8/50 (16)
TCR positive	26/41 (63)

Conclusion. Clonal T-LGL disorders in patients with chronic neutropenia are not rare and do not account for serious cytopenias or infectious complications. More longitudinal studies could clarify the clinical course and the need for treatment of T-LGL clonal disorders found in patients with chronic neutropenia.

0785

B-CELL COUNT AND TIME TO FIRST TREAMENT: DIFFERENTIATING CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) FROM MONOCLONAL B-CELL LYMPHOCYTOSIS (MBL) BASED ON CLINICAL OUTCOME: THE GIMEMA EXPERIENCE.

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Rather than using the traditional but arbitrarily derived 5×10^9/L B-lymphocyte threshold, generally utilized to differentiate monoclonal B-cell lymphocytosis (MBL) from chronic lymphocytic leykemia (CLL), our study includes all patients with isolated lymphocytosis to determine which lymphocyte threshold best predicts clinical outcome. To this purpose we analyzed the relationship of either absolute lymphocyte count [ALC] or B-cell count with the clinical outcome in 818 consecutive CLL patients with Rai stage 0 (i.e., no palpable lymphadenopathy or hepatosplenomegaly) who had flow cytometry evaluation at the time of diagnosis and were included in a GIMEMA (Gruppo Italiano Malattie EMatologiche dell'Adulto) database. In this patient cohort, both ALC (P<0.0001) and B-cell count (P<0.0001) when treated as continuous variables (i.e., measuring the risk of a 1.0×10^9/L increase in the cell count) related to time to first treatment (TFT). However, in a Cox multivariate only the B-cell count retained its discriminating power (P<0.0001). Receiver Operating Characteristic (ROC) analysis identified an ALC ≥ 11.5×10^9/L (P<0.0001) and an absolute B-cell lymphocyte count ≥10.0×10^9/L (P<0.0001) as the best thresholds separating patients who subsequently required treatment from those with stable disease. When overall survival was used as endpoint, only a trend towards a longer survival for patients with a B-cell count <10.0×10^9/L could be found (P=0.06). Finally, the B-cell threshold used in the current diagnostic criteria (i.e., 5×10^9/L) was not able to predict both TFT (P=NS) and overall survival (P=0.23). Our results provide a clear justification to retain the B-cell count as the reference gold standard of CLL diagnosis, but imply that a count of 10×10^9/L B-cell is the best lymphocyte threshold predicting TFT. The use of clinical outcome to distinguish CLL from other premalignant conditions, such as MBL, is a pragmatic approach that meets the requirement of reducing patients' psychological discomfort receiving a diagnosis of leukemia when the risk of adverse clinical consequences is low.

0786

IN VITRO ACTIVITY OF THE CD20 ANTIBODY OFATUMUMAB IN GENETIC HIGH-RISK CLL WITH TP53 MUTATION/DELETION

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Introduction. CD20 represents a well-established target for immunotherapy of B-cell malignancies. Relatively little is known about activity of different CD20 antibodies in genetic subgroups of chronic lymphocytic leukemia (CLL). Ofatumumab (OFA) is a novel human CD20 monoclonal antibody, which binds a unique membrane-proximal epitope encompassing both the large and small loop on the CD20 molecule. The FDA recently approved OFA (Arzerra), for the treatment of fludarabine-(F) and alemtuzumab (ALM)-refractory CLL. The aim of the current study was to assess the *in vitro* activity of OFA in a group of genetically characterized CLL cases to investigate whether its activity is impacted by TP53 status. *Methods.* To assess the activity of OFA *in vitro*, we studied B cell depletion in a set of CLL patients (n=18), in the presence of serum. CLL samples were characterized with respect to genetics (genomic aberrations, TP53 mutation, IGHV mutation status), clinical course and immunophenotype. The effects of OFA were compared to RTX, ALM and F as references. At different time points (3h, 48h), cell death was measured by use of flow cytometry (staining with 7-AAD + Annexin-PE) and Cell Titer Glo Luminescent Assay. *Results.* OFA induced dose-dependent (0.1-100 μg/mL) cell death and thereby reduced viability to a maximum of 44.5% (n=18; % of viable cells after 3 hrs incubation with 100 μg/mL OFA) as compared to control. At 10 and 100 μg/mL OFA was more effective at killing CLL cells than RTX (10 μg: 63.7% viable cells (OFA) *vs.* 98.8% viable cells (RTX); 100μg 44.5% viable cells (OFA) *vs.* 95.3% viable cells (RTX) (P<0.001). The combination of F (1 μM) with OFA (1 μg/mL) added to the induction of cell death (48h: F: 49.6% viable cells; F+OFA 35.9% viable cells; F+RTX: 41% viable cells). To investigate the effect of OFA on CLL cells carrying a TP53 deletion/mutation, we separated the cohort into two groups, with (n=6) or without (n= 12) TP53 mutation/deletion. In the cohort without TP53 defects, OFA induced CLL cell death in a dose-dependent manner (10 μg: 61% viable cells (OFA) *vs.* 99% viable cells (RTX) (P<0.001); 100 μg: 41.5% viable cells (OFA) *vs.* 92.2% viable cells (RTX) (P<0.001)) (Figure1). In the group with TP53 defects, OFA, interestingly, also showed a dose-dependent effect (Figure 1), and, again, OFA was significantly more potent than RTX (10 μg: 69.1% viable cells (OFA) *vs.* 98.8% viable cells (RTX) (P=0.014); 100 μg 50.6% viable cells (OFA) *vs.* 101.4% viable cells (RTX) (P=0.001)). Furthermore, the response to F at 48 hours was lower in the group with TP53 defects (74.1% viable cells), as compared to the response to F in the group without TP53 defects (36.3%), which is in keeping with the well known association of these aberrations with chemorefractory CLL. *Conclusion.* Compared to RTX which represents the currently most widely used mAb in CLL, OFA appears more potent at equivalent concentration in depleting CLL cells in a CDC assay. Significantly, this activity of OFA appears to be independent of high risk genetic subgroups as cell killing is of similar magnitude in TP53 wild type *vs.* mutated cells.

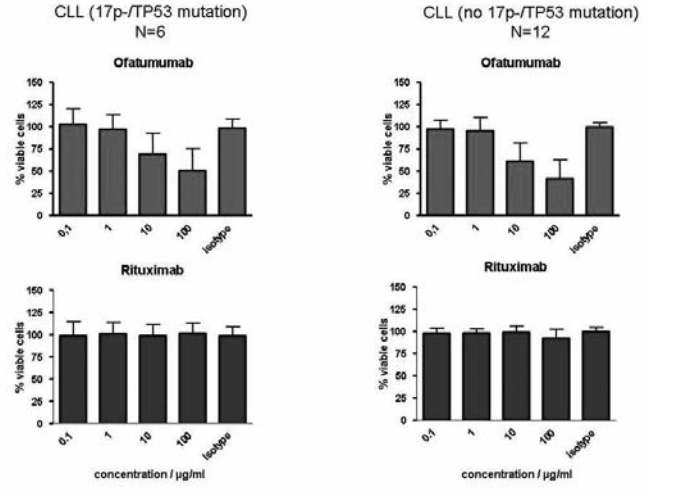

Figure 1. Induction of cell death by Ofatumumab (*in vitro*).

0787

GENETIC FEATURES OF CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS WITH AUTOIMMUNE HEMOLYTIC ANEMIA: VDJ-REARRANGEMENTS AND CHROMOSOMAL ABERRATIONS

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Background. Autoimmune hemolytic anemia (AIHA) is a severe complication during the course of chronic lymphocytic leukemia (CLL). There is little known about triggers and predicting markers for this phenomenon. *Aim.* Evaluate the incidence of AIHA in a unicentric cohort of 548 CLL patients and examine the role of VDJ-rearrangement structure and chromosomal aberrations in the AIHA subgroup. *Methods.* IGHV analysis by PCR plus direct sequencing and evaluation of chromosomal aberrations by FISH in patients with CLL and AIHA. *Results.* 10% (55/548) of the CLL cohort showed an AIHA. 20/55 were diagnosed by standard criteria alone (drop in hemoglobin, increased reticulocyte count and rise in indirect bilirubin with no other cause for anemia identified) whereas 35/55 cases also showed a positive direct antiglobin test. Male gender was significantly overrepresented in the AIHA group. (43/55 *vs.* 295/493; p-value: 0,0082) IGHV analysis was available in 49 patients with a significant overrepresentation of IGHV-unmutated cases 40/49 (18,4%; P: 0,0006). VH1-69, representing the most frequent VH-gene in the AIHA-CLL cases (12/49 = 24,5 %) was significantly overrepresented (P 0.0198) compared to the 446 non-AIHA-CLL cases (57/446 = 14,3%) with available IGHV-data. VH4-34 detected in 8% of all non-AIHA-CLL cases in our analysis was underrepresented with only one single rearrangement in 49 AIHA cases. The analysis of the HCDR3-structure of the AIHA-CLL cases revealed some interesting findings: 2/49 cases showed the identical VDJ-combination VH1-69/D3-16/JH3b with 100% VH-homology and a 21 amino acid (aa) HCDR3 with the highly homologous HCDR3-motif: ARGXXY-DYVWGSYRXNDAFDI (= stereotype 1) In the non-AIHA group the same HCDR3 structure was not detected in a single case (p-value: 0,0096). In our data collection of 4942 multicentric CLL patients with no clinical data available stereotype 1 was detected in only 10/4942 cases (P<0.0001) . Another 2 cases rearranged VH1-69/D2-2/JH6b with 100% VH-homology and a 22 aa HCDR3 with the HCDR3-motif: ARVXPDIVVPAXXXYYYGMDV (= stereotype 2) whereas in the non-AIHA group this stereotype was also not detected in a single case (p-value: 0,0096) and in only 3 of the 4942 collected CLL cases. (P<0.0001). Finally 2 cases showed the highly homologous HCDR3-motif: ARE-QWLXTMXFDY (= stereotype 3) with either VH1-03 or VH1-18 in combination with D6-19 and JH4b, 100% VH-homology and a 13 aa HCDR3. Again non of the non-AIHA-CLL cases and only 2/4942 collected CLL cases showed the same HCDR3-motif. (p-value <0,0001). For 53 AIHA-CLL cases and 371 non-AIHA-CLL cases FISH analysis was performed: The high risk aberrations deletion-11q and deletion-17p are significantly overrepresented compared to the non-AIHA-CLL cases (11q: 15/53 *vs.* 65/371; 17p: 9/53 *vs.* 29/371). *Conlusions.* AIHA is a frequent phenomenon in CLL (~ 10%) with characteristic genetic features. In our analysis AIHA-CLL cases were characterized by a significant overrepresentation of unfavourable prognostic markers like unmutated IGHV-status and high risk chromosomal aberrations. Beyond that the overrepresentation of stereotyped HCDR3-motifs especially using the VH1-69 gene could be a clue to antigentriggered mechanisms in those patients. A more detailed analysis of the whole B-cell receptor including the light chain structure should follow.

0788

PREDICTIVE POWER FOR OVERALL SURVIVAL OF CD38, ZAP-70, DELETIONS OF 17P OR 11Q AND IG MUTATIONS: MULTIVARIATE ANALYSIS ON 747 CASES OF CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. We showed previously that a multivariate model based on age, stage, beta2microglobulin levels and sex was able to predict survival in chronic lymphocytic leukemia (CLL). However, expression of ZAP-70 and CD38, deletion of 17p- or 11q- by FISH and immunoglobulin mutational status (IgVH) are also frequently evaluated in untreated CLL patients for better prognostication. Despite this, little is known on the relative importance of each o f these biological predictors or about their actual capability to add independent predictive power to a simple scoring system as that developed by us. Aim. To test the independent predictive power of ZAP-70, CD38, 17p-/11q-, IgVH in a multivariate model adjusted for a 3 level stratification scoring based on sex, age, stage and beta2microglobulin. *Methods.* We developed a prognostic score for predicting overall survival in 1037 CLL patients recruited in 8 Italian and Swiss-Italian centers. The scoring was based on age (0 points ≤ 60, 1 point 61-70, 3 points 71-80, 7 points >80), Binet stage (0 points A, 3 points B or C), sex (2 points male, 0 points female) and standardized bet2microglobulin (0 points ≤1, 1 point >1 and ≤2, 2 points >2). The beta2microglobulin value was divided by its upper reference limit. All the variables were measured at or within 1 year from diagnosis. In a subset of 747 patients with complete data, CD38, ZAP-70, 17p-/11q- and IgVH were each tested by unadjusted Cox models or adjusted for prognostic score. A P≤0.05 was considered statistically significant. Results. Three risk groups were stratified by the prognostic score: low risk (32% of patients) with score ≤2; intermediate risk (50%) with 2<score≤6; high risk (18%) with score >6. Overall survival at 10 years was respectively 92.3, 72.0 and 38.2%. Univariate (unadjusted) hazard ratio of the biological prognosticators increased from 1.44 of ZAP-70 (with borderline p), to 1.77 of CD38, 2.0 of IgVH, 2.38 of 17p-/11q- (Table 1).

Table 1.

	Unadjusted		Adjusted for prognostic score	
predictor	HR	p	HR	p
ZAP-70>20%	1.44	0.077	1.18	0.41
CD38>30%	1.77	0.0063	1.50	0.056
IgVH UM	2.0	0.00076	1.75	0.007
17p-/11q-	2.38	0.000057	2.11	0.00059

In a model adjusted for the 3 level prognostic score, ZAP-70 was not an independent predictor, CD38 was at borderline significance, IgVH

and 17p-/11q- kept to be significant (Table 1). In a further model we simultaneously analyzed CD38, IgVH, 17p-/11q- and the prognostic score. Only 17p- and the prognostic score were significant independent predictors. *Summary/Conclusions.* A simple scoring using clinical data (sex, age, Binet staging) and the easily measurable β 2μg, was able to stratify CLL patients in three groups with different clinical risk. CD38, IgVH and 17p-/11q- could retain prognostic power when adjusted with this prognostic score, but not ZAP-70. This could reflect the lack of standardization in the measurement of this intracytoplasmic antigen, a fact possibly emphasized in a multicenter collection of cases. Moreover, both CD38 and IgVH were not independent in multivariate analysis, the latter with a borderline value (P=0.078, HR 1.47). Larger sample or an higher number of death events are required to overcome these discrepancies.

0789

DIAGNOSTIC AND THERAPEUTIC APPROACH TO SMALL LYMPHOCYTIC LYMPHOMA (SLL): EXPERIENCE OF CZECH LYMPHOMA STUDY GROUP

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Background. Small lymphocytic lymphoma (SLL) is an indolent lymphoproliferation comprising 3-10% of Non-Hodgkin´s lymphomas (NHL). WHO 2008 classification of lymphoid neoplasms lists SLL together with chronic lymphocytic leukemia (CLL); these entities differ by distribution of tumor load and arbitrary cut-off of lymphocytosis 5×10^9/L. Clinical course of SLL is still relatively ill-defined due to lack of published data because clinical studies in CLL usually exclude patients with SLL. This results in considerable variability in diagnostic and therapeutic approach in daily practice. Aims of the study. to perform a retrospective analysis of patients (pts) with SLL with emphasis on diagnostic procedures and management in real life. *Methods.* We analyzed consecutive patients from 16 centers participating within the Czech Lymphoma Study Group who fulfilled diagnostic criteria for SLL, i.e. coexpression of CD5/C19/23 by flow cytometry or typical histology and immunohistochemistry with peripheral blood lymphocytes $<5\times10^9$/L. *Results.* As of April 2008, data were available on 157 patients (63% males with median age 66 years range, [29-87]). Ann Arbor stage I/II/III/IV was present in 6/3/6/85 %. Splenomegaly was detected in 18%, elevated lactate dehydrogenase (LDH) in 30 % and bone marrow involvement in 79% of pts. Of note, 94% pts underwent computer tomography (CT) of thorax and abdomen and 86% bone marrow biopsy at initial staging. Mediastinal and retroperitoneal lymphadenopathy was discovered in 42% and 68%. Generalized lymphadenopathy (more than 4 involved regions) was found in 49%. Bulky lymphadenopathy (>5 cm) was present in 38%. Thirty per cent of pts had B-symptoms. Data regarding treatment were available on 145 pts; was initiated in 132 (91%) pts. Systemic treatment was used in 123 pts: chemotherapy, n=79; chemoimmunotherapy, n=51 (rituximab, n=50 pts; alemtuzumab, n=1). Furthemore, 11 pts were treated by radiotherapy and 5 by surgery. Surprisingly, only 13 pts were managed using the watch and wait strategy. The most frequently used regimens in first line were CVP (19%), FCR (15%), R-CVP (14%), CHOP (13%), chlorambucil (11%), FC (10%) and R-CHOP (10%). At the median follow-up of 38 months (mo), median overall survival (OS) was 78 mo. OS was significantly influenced by splenomegaly, B-symptoms, IPI, response to therapy, type of treatment (fludarabine-based *vs.* anthracycline-based *vs.* alkylator-based) and use of rituximab. However, in multivariate analysis, only use of rituximab, type of treatment and quality of therapeutic response retained prognostic significance. *Conclusions.* We confirmed previously published epidemiological data on SLL. In contrast to CLL (where routine radiological assessment is not recommended by NCI-WG criteria), nearly all SLL patients underwent CT for staging; however, use of radiological assessment seemed justified as more than two thirds of pts had significant abdominal lymphadenopathy. Therapeutic approach varied considerably; majority of pts were treated by "lymphoma" regimens but fludarabine-based protocols yielded the best *Results.* In our opinion, SLL should be managed identically to CLL but imaging methods appear to be valuable for appropriate staging. Our study underscores the need for standardized diagnostic and therapeutic approach to SLL.

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0790

INTEGRATION OF TOTAL BODY CT SCAN IN THE INITIAL WORK-UP OF BINET STAGE A CLL PATIENTS ON CLINICAL GROUNDS: PRELIMINARY RESULTS OF A PROSPECTIVE, MULTICENTER OBSERVATIONAL-CLL1-GISL STUDY

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Background. In contrast with other chronic lymphoproliferative disorders, CT scans are not routinely recommended for routine staging CLL patients and there are no studies investigating the clinical significance of integration of total body CT in the initial work-up of CLL patients. *Aims.* We investigated whether total body CT allowed to discriminate among Binet stage A CLL patients on clinical grounds, cases in more advanced stage and whether this subgroup showed a different expression of clinical and biological prognostic markers. Patients & Methods. To date, 380 patients have been enrolled in the trial started in April 2007 and total body CT scan were available for 127 patients. All patients are in Binet stage A, while 91 patients were at low risk (0 stage) and 36 at intermediate risk (I-II stage) by Rai classification. The median age was 60.5 years (range, 33 to 70 years) and 60% were male. Thirty-six% of cases were IgVH unmutated, 42.5% had a high ZAP-70 expression and 22% were CD38 positive (>30%). LDH and B2-microglobulin were elevated in 11% and in 28% of cases, respectively. FISH data are available for 116/127 cases; 53 patients (46%) showed del(13q14), 14 (12%) trisomy 12, 7 (6%) del(11q22.3) and 4 (3%) del(17p13), 38 cases (33%) were normal. Cytogenetic abnormalities were clustered in 3 risk groups [i.e. low (del(13q14) and normal), intermediate (trisomy 12) and high risk del(11q22) and del(17p13)] as suggested by others. Following international standard criteria, lymph nodes

more than 10 mm in diameter were considered abnormal. The abdominal lymphoid areas (diaphragmatic, celiac, mesenteric, iliac, and retroperitoneal) were considered as single lymph-node region, while spleen and/or hepatic enlargement was considered separately. *Results.* Considering total body CT scan, 40/127 (41.5%) patients were re-considered as Binet stage B. Notably, 70% were male, LDH and B2-microglobulin were elevated in 8% and in 34% of cases respectively, 45% were IgVH unmutated, 47.5% had a high ZAP-70 expression, 32.5% were CD38 positive, 16% showed a high-risk FISH. Both main clinical characteristics and biological prognostic markers failed to correlate with a more advanced stage. In fact, no statistically different distribution of gender, age, LDH and β2-microglobulin, such as IgVH mutational status, CD38 or ZAP-70 expression and cytogenetic abnormalities were observed between Binet A and Binet B cases. According to the Rai classification 35/91 (38.5%) low risk patients became at intermediate risk with the integration of total body CT scan. In this subset of patients a statistically significant higher rate of cases with elevated CD38 expression than patients at low risk (P=0.019) was observed. Finally, total body CT scan allowed the early detection of a second neoplasia in 2 cases (lung cancer 1 pt, renal cell carcinoma 1 pt). Conclusion. Our results indicate that the integration of total body CT in clinical staging discriminates within Binet A CLL cases about 40% of patients with a more advanced stage. A longer follow-up will aid in demonstrating whether the inclusion of total body CT scan in the initial work-up of patients in clinical early-stage provides relevant prognostic information.

0791

MODULATION OF CD20 ANTIGEN EXPRESSION IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA AFTER RITUXIMAB THERAPY

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Background. Immunotherapy with CD20-targeting antibody (rituximab) in combination with chemotherapy has significantly improved the survival of patients with lymphoproliferative disorders (LPD), in particular of those with follicular and diffuse large B-cell non-Hodgkin's lymphomas (NHL). In addition, rituximab-containing regimens are now also considered a standard approach for B-cell chronic lymphocytic leukemia (CLL). CD20 antigen down-modulation by anti-CD20 rituximab therapy is a well recognized phenomenon in patients with NHL. However, a few data are currently available on this topic in CLL. *Aims.* Aiming to evaluate the frequency and the clinical significance of the CD20 disappearance on neoplastic cells, we analysed by flow cytometry the CD20 expression in patients affected by CLL treated with rituximab in combinations with other agents. In this setting, that here reported is, to the best of our knowledge, the largest series of CLL so far sequentially analysed. Patients and *Methods.* The surface expression of CD20, CD19, CD5, CD23, CD10, CD22, CD52, FMC7, CD38, kappa and lambda light chains was studied on marrow neoplastic cells by flow cytometer in 11 CLL, and, for comparison, in 4 follicular lymphomas, 3 mantle cell lymphomas, 1 marginal zone lymphoma and 3 otherwise unspecified "indolent"LPD treated with various combinations of rituximab and other cytostatic agents. *Results.* Overall, nine patients (40,9%) achieved a stable complete remission and did not show flow cytometry evidence of CD20-positive neoplastic cells in post-therapy samples. Thirteen patients maintained instead a variable proportion of B clonal cells after rituximab treatment; among them, four CLL (18,2% of the entire population analyzed, 36.3% of all CLL) evidenced CD20 disappearance on residual marrow neoplastic cells after salvage therapies including rituximab (Figure 1). In particular, CD20 antigen was lost in two patients in partial response after R-CHOP-like regimen and chlorambucil/rituximab combination, respectively. Both these patients relapsed 5 to 13 months after their last dose of rituximab, showing reappearance, however, of a CD20-positive phenotype at progression. A third patient did not respond to R-Flu/Cy, showing persistence of about 50% of marrow clonal CD20-negative B cells. CT and PET scans indicated disease progression and lymph-node biopsy documented a transformation toward a high grade, CD20-positive non-Hodgkin lymphoma (Richter's syndrome). The patient died soon after. The last patient received R-CHOP-14 therapy after a biopsy-proven high grade CD20-positive lymphoma transformation (Richter's syndrome). A transient partial response was achieved; however, at this time, residual bone marrow CLL cells infiltration had lost CD20 surface antigen. After further progression, additional treatments, including plerixafor mobilized peripheral blood autologous stem cell transplantation, were ineffective and the patient died. *Conclusion.* In our experience, loss of CD20 was seen in more than one third of CLL patients receiving rituximab therapy. This phenomenon may be transient and clinically heterogeneous. However, a possible relationship with evolution into high grade NHL transformation needs to be further investigated. In fact, Richter's syndrome was observed in 2 out of our 4 CLL patients who lacked CD20 expression, but only in one case out of all other 49 CLL we have followed at our Institution during the last four years.

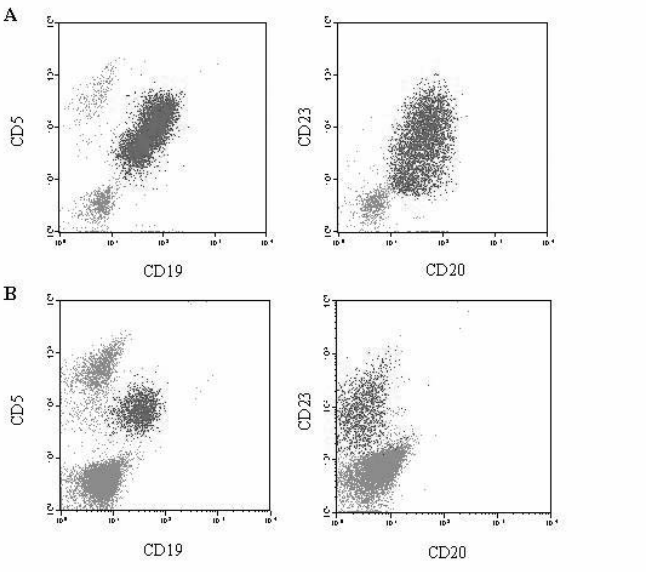

Figure 1. Flow cytometry analysis of bone marrow leukemic cells before (A) and after (B) rituximab therapy showing cpmplete loss of CD20 antigen expression, while CD19, CD5 and CD23 were stillpositive (patient 4).

0792

EFFICACY OF FC AND FCR REGIMENS IN THE FIRST LINE TREATMENT OF PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA: 7,5 YEARS OF FOLLOW-UP

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Background. Fludarabine-containing regimens are widely used as the first-line treatment for CLL. Recently phase III trial comparing fludarabine and cyclophosphamide alone (FC) with combination of fludarabine, cyclophosphamide and rituximab (FCR) showed significantly superior results in FCR arm in terms of response rates, PFS and OS. Thus this particular chemoimmunotherapy regimen may become standard for initial CLL treatment. However effectiveness of this treatment approach needs to be confirmed in everyday clinical practice. *Aims.* To estimate survival benefit produced by the addition of rituximab to standard FC regimen for treatment-naïve not selected CLL pts. *Methods.* 77 consecutive pts were included (43 in FC and 34 in FCR arm) between 2002 and 2008 according to intent-to-treatment. Informed consent was obtained in each case. Assignment to FCR was based predominantly on the availability of rituximab in general practice and nearly all newer pts received it as part of treatment. Arms were well balanced with regard to sex (M-to-F ratio ~2:1) and age (median 59 years, range 43-78) though there was tendency towards more advanced disease stage in FC arm (15 *vs.* 7 pts BinetC). Treatment schedules were standard. Treatment was suspended or withdrawn in case of severe toxicity, no dose reduction was done. Only 11 pts received less than 6 cycles (range 4-5). Response was defined according to NCI criteria. CD38 was measured by cytoflow with 30% cut-off (available in 57 pts). VH genes were analyzed by cDNA sequencing with 98% threshold (available in 32 pts). Statistical analysis was performed in SPSS17. *Results.* OR and CR rates in FCR and FC groups were 91% *vs.* 81% (P=0,18) and 67% *vs.* 46% (P=0,05) respectively. PR rates were 24% for FCR and 35% for FC (P=0,2). 3-year OS in FC arm was 54%, compared to 76% in FCR arm based on analy-

sis of 66 pts. Estimated 4-year survival was 45% in FC and 66% in FCR arm (61 pts analysed). After 7,5 years of follow-up there were 39 events in FC (90,7%) and 17 in FCR arm (50%). 27 deaths were registered in FC arm (62,8%) and 8 in FCR (23,5%). Median PFS in FCR arm was 31 mos compared to 19 mos in FC arm (P=0.003), median OS in FCR is n.r. and 45 mos in FC group (P=0.011). CD38 status didn't predict PFS and OS in FCR arm. In FC arm pts with high expression of CD38 had inferior median PFS (19 *vs.* 32 mos, P=0,039) and a tendency towards shorter median OS (40 mos *vs.* n.r., P=0,06). Median PFS was inferior in VH-unmutated cases (52 *vs.* 23 mos, P=0,05). There was also tendency towards inferior OS in this group of pts (n.r. *vs.* 42 mos., P=0,08). Conclusion. FCR regimen has advantage over FC regimen in terms of frequency of induced OR and CR. 3-year survival of pts treated with FCR is nearly 2-fold higher compared to FC alone. Addition of rituximab to FC regimen in first line improves OS and PFS. We recommend FCR regimen as first-line CLL treatment in general clinical practice.

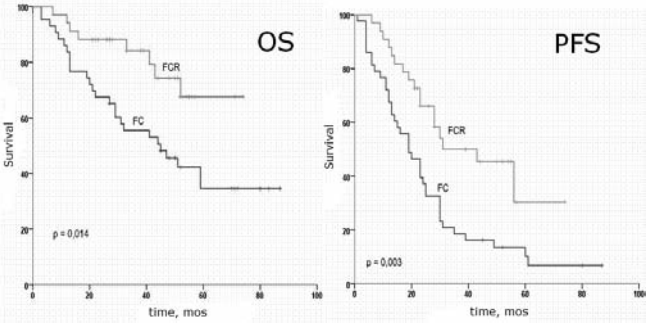

Figure 1. OS and PFS in FC vs. FCR.

Chronic myeloid leukemia - Biology 2

0793

BORTEZOMIB DECREASES RB PHOSPHORYLATION IN IMATINIB-SENSITIVE AND -RESISTANT BCR-ABL1 EXPRESSING CELLS

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Background. Chronic Myeloid Leukaemia (CML), resulting from the expression of the oncoprotein Bcr-Abl1, is usually treated with the specific kinase inhibitor Imatinib. However, point mutations in Bcr-Abl confer resistance to this treatment. We have previously shown that Bcr-Abl1 induces the proteasomal degradation of proteins relevant for cell cycle control, such as p27Kip1. Therefore, we proposed the use of proteasome inhibitors in the treatment of the disease. *Aim.* In this study we wanted to evaluate the effect of the proteasome inhibitor Bortezomib on the proliferation and survival of Bcr-Abl1 expressing cells, including mutant forms that show resistance to Imatinib, and analyse the signal transduction pathways involved. Materials and *Methods.* We have used the BaF/3 cell line and BaF/3 cells expressing wt Bcr-Abl (BaF/3-wt) and different Imatinib resistant mutants of the protein, including the highly resistant mutant T315I (BaF/3-T315I). We have treated these cells with Bortezomib and analysed its effects on cell proliferation and survival by flow cytometry. We have also analysed by western blot the expression and activity of different proteins involved in cell cycle regulation. *Results.* Proteasome inhibition with Bortezomib inhibits growth of BaF/3 cells, but Bcr-Abl1 expressing cells (BaF/3-p210) are more sensitive than the parental BaF/3 control cells. At the dose of 6nM, Bortezomib induces an irreversible cell cycle arrest at G1 only in BaF/3-p210 cells and, later on, cell death by apoptosis. This effect is also seen in BaF/3 cells expressing different Imatinib-resistant mutants of Bcr-Abl1 (Q252H, Y253F, E255K and T315I). At the molecular level, the cell cycle arrest correlates with an accumulation of p27Kip1 and a decrease in the phosphorylation of Rb. By Western-blot, we have analysed the activation of p52 NF-kB, which is processed from p100 to p52 in a proteasome dependent manner. BaF/3-p210 cells show higher levels of active p52 NF-kB than BaF/3 control cells and the treatment with Bortezomib results in a decrease of the active p52 processed form and an increase of the inactive p100 form of the protein. Moreover, two targets of p52 NF-kB that are relevant for the regulation of p27Kip1, such as c-Myc and Skp2, show reduced levels of expression in Bortezomib treated BaF/3-p210 cells, suggesting that in these cells the non-canonical NF-kB pathway may be a target of Bortezomib. Finally, we also show that primary $CD34^+$ cells from a CML patient are more sensitive to Bortezomib than non-CML primary $CD34^+$ cells. *Conclusions.* Our data show that Bortezomib treatment of Bcr-Abl1 expressing cells results in a G1 cell cycle arrest and apoptosis, with a decrease of Rb phosphorylation and caspase activation. Furthermore, BaF/3 cells expressing Bcr-Abl1 mutants resistant to Imatinib are also sensitive to Bortezomib. These results unravel a new molecular target of Bortezomib, i.e. the Rb pathway, and open new possibilities in the treatment of CML especially for patients that become resistant to Imatinib due to the presence of the T315I mutation.

0794

VARIANT PH TRANSLOCATION IN EARLY CHRONIC PHASE OF CHRONIC MYELOID LEUKEMIA: CYTOGENETIC - MOLECULAR CHARACTERIZATION AND CORRELATION TO IMATINIB MESYLATE THERAPY (A GIMEMA WP ON CML ANALYSIS)

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Background. At diagnosis, variant Philadelphia (Ph) translocation occurring in Chronic Myeloid Leukemia (CML), have been reported in 5-10% of patients (patients). Some studies have reported no impact on prognosis after Imatinib Mesylate (IM) treatment. Variant translocation could be three or four-way translocation (involving chromosomes 9, 22 and 1 or 2 additional chromosomes respectively), associated or not with deletions of ABL1, BCR or ABL1-BCR. Two possible mechanisms of generation of these variant translocations have been hypothesized: a one-step mechanism wherein chromosome breakage was on three different chromosomes simultaneously, or a two-step mechanism involving two sequential translocations, in which a standard t(9;22) translocation is followed by subsequent translocation involving additional chromosomes. *Aim.* To investigate the role of occurrence of variant translocations on the response to IM in early chronic phase (CP) CML patients. *Methods.* A sub-analysis of 533 evaluable CML patients in early CP, have been performed within 3 simultaneously running trials of the GIMEMA WP on CML (CML/021; CML/022; CML/023). Median observation time was 42 months. Monitoring: hematologic, continuously; CC, FISH and molecular analysis were performed at baseline, 3, 6, and 12 months and then every 6 months by local or reference labs. *Results.* At enrolment, 30 patients (5.6%) had variant translocation: 2 patients (6.7%) had a four-way translocation; 28 patients (93%) had a three-way translocation. We analyzed 24 patients: 18 patients showed a one-step mechanism, 4 a two-step and 2 a multistep. In 3 patients (10%) translocation was associated with deletion of ABL1, in 2 patients (6.7%) with deletion of BCR and in one patient (3.3%) with deletion of ABL1-BCR; in 2 cases deletion was on third chromosome involved and it has been characterized by FISH and SNPs assays. One case was Ph masked and characterized by multistep mechanism. Another one carried an additional chromosome abnormality: t(7;19)(q21;p13). Patients with or without variant translocation were similar for age, Sokal risk and IM dose. During follow-up, 25 patients achieved complete cytogenetic response (CCgR; 83.3% *vs.* 98.1% in patients without variant) and major molecular response (83.3% *vs.* 83.2%); in 6 treatment was unsuccessful (20% *vs.* 18.1%). The 2 patients with four-way translocation and 4 of 6 patients (66.7%) with deletions reached CCgR. *Conclusions.* In the present large series of early CP patients treated with IM therapy, we found no difference in cytogenetic and molecular response rates between cases with or without variant translocations. Some studies suggested that the formation of variant translocations (in particular those with two-step mechanism) was similar to or was in essence a clonal evolution, and consequently associated with poorer prognosis. Our series of patients did not show similar association.

0795

CML STEM CELLS (CD34+/CD38-) EXPRESS SIGLEC-3/CD33 AND RESPOND TO THE CD33-TARGETED DRUG GEMTUZUMAB/OZOGAMICIN

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Siglec-3 (CD33) is an established therapeutic target in acute myeloid leukemia (AML). We and others have shown that CD33 is expressed on immature $CD34^+/CD38^-$ stem/progenitor cells in AML. Here, we describe that leukemic progenitor cells obtained from patients with chronic myeloid leukemia (CML) display high levels of CD33, and that CD33 may serve as a potential target in CML. As assessed by multi-color flow cytometry, $CD34^+/CD38^-/CD123^+$ CML stem cells were found to express 5-10 fold higher levels of CD33 compared to normal $CD34^+/CD38^-$ bone marrow stem cells. In chronic phase (CP) CML, CD33 was found to be expressed homogeneously on most or all $CD34^+/CD38^-$ stem cells. In patients with accelerated (AP) or myeloid blast phase (BP), CML stem cells also co-expressed CD33, but the levels of CD33 varied from donor to donor, and in one of these patients, most CML stem cells appeared to be CD33-negative. In two patients with CML, $CD34^+/CD38^-$ cells were highly enriched by cell sorting (purity >98%) and found to contain CD33 mRNA in qPCR analysis. Expression of CD33 was also demonstrable in more mature myeloid CML progenitor cells in all patients. We also examined the effects of the CD33-targeted drug gemtuzumab/ozogamicin (GO) on growth of primary CML cells. As assessed by 3H-thymidine uptake, GO produced growth inhibition in leukemic cells in all patients tested (CP, n=7; AP, n=2). The effects of GO on leukemic cell growth were dose-dependent and occurred at low dose, with IC_{50} values ranging between 1 and 50 ng/mL. In one patient with imatinib-resistant CML in BP, we were also able to determine the effects of GO on survival of leukemic stem cells. As assessed by Annexin-V staining, GO was found to induce apoptosis in $CD34^+/CD38^-$ CML stem cells. In conclusion, CD33 is expressed abundantly on $CD34^+/CD38^-$ stem cells in CML. Whether GO can be employed to eradicate residual leukemic stem cells in CML CP patients remains at present unknown.

0796

CORRELATION BETWEEN GENETIC POLYMORPHISMS OF THE MDR1, HOCT1 AND ORM1 GENES AND THE RESPONSE TO IMATINIB IN PATIENTS WITH NEWLY-DIAGNOSED CHRONIC-PHASE CHRONIC MYELOID LEUKEMIA (CP-CML)

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Background. Imatinib (IM), a selective inhibitor of the Bcr/Abl tyrosine kinase, is the current frontline treatment of CP-CML. Although most patients respond to IM, some do not have an adequate response, either primarily or secondarily. In such cases, several mechanisms of resistance have been described, the better characterized being the presence of mutations in the BCR/ABL kinase domain. Recently, the possible impact on the response to IM of polymorphisms in genes encoding for proteins involved in IM transportation has been pointed out. However, the results are controversial. *Aim.* To assess the role in the inadequate response to IM of single nucleotide polymorphisms (SNPs) in the genes OCT1 (codifying for the IM influx protein OCT1), MDR1 (involved in IM efflux), and ORM1 (that codifies a plasma protein binding to different drugs), all of which are involved in IM bioavailability. *Methods.* Sixty-five patients newly diagnosed with CP-CML treated upfront with IM 400 mg daily were studied. Informed consent was obtained. Allelic discrimination by RQ-PCR (ABI Prism 7900; TaqMan probe, Applied Biosystems) of 8 SNPs in OCT1 (rs6935207, rs628031, rs12208357, rs72552763), MDR1 (rs1128503, rs2032582, rs1045642), and ORM1 (Gln38Arg) genes was performed. Genotypes were correlated with the patients' main characteristics at diagnosis and the response to treatment (either optimal, suboptimal or resistance), which was defined according to the recommendations of the European LeukemiaNet (Baccarani *et al.*, J Clin Oncol 2009). For the purpose of the present analysis, suboptimal response plus resistance was considered as inadequate response. In order to rule out other potential causes of inadequate response to IM, bone marrow FISH analysis (to exclude BCR/ABL amplification) and direct sequencing mutational study of the BCR/ABL kinase domain were also performed. *Results.* Patients' median age was 47 (range: 19-75) years; 39 were males. Distribution by Sokal groups was: low risk, n=29; intermediate risk, n=25; and high risk, n=11. Median follow-up was 60 months. Fifteen patients (23%) had primary suboptimal response to IM and 5 (8%) were primarily resistant, for an overall primary inadequate response rate of 31%. Nine patients developed secondary suboptimal response and 3 became resistant. Genotype frequencies observed in the 65 patients were comparable to those reported in a healthy Caucasian population. When the influence of the genetic polymorphisms in the therapeutic response to IM was analyzed, the presence of the CC genotype in the rs1045642 (C3435T, Ile1145Ile)

MDR1 SNP was found to be associated with a higher probability of primary inadequate response (P=0.05). Mutations were detected in 3 patients, but in only one at the time of primary resistance to IM; BCR/ABL amplification was not found in any case. At multivariate analysis, which included the patients' main initial features, Sokal risk group and SNPs genotype, the only factor associated with a primary inadequate response to IM was the Ile1145Ile MDR1 polymorphism (RR=4.118; CI 1.027-16.509; P=0.046). *Conclusion.* In newly-diagnosed CP-CML, the Ile1145Ile MDR1 polymorphism has predictive value for the response to standard dose IM. Patients with CC genotype for this polymorphism have a higher probability of primary inadequate response to IM.

0797

CELLULAR DISTRIBUTION OF HEPATOCYTE GROWTH FACTOR (HGF) AND HGF RECEPTOR (C-MET) IN CHRONIC MYELOID LEUKEMIA

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Chronic myeloid leukemia (CML) is a myeloproliferative disease in which BCR/ABL leads to enhanced survival of leukemic cells. Several different angiogenic molecules, including vascular endothelial growth factor (VEGF), have been implicated in the pathogenesis of CML. Hepatocyte growth factor (HGF) is a key mediator of vascularization in normal and neoplastic tissues and has recently been described to be expressed in CML cells. In the present study, the cellular distribution of HGF and its receptor c-MET, and the mechanism of expression of HGF in CML cells were examined. As assessed by immunostaining of bone marrow sections and isolated blood and bone marrow cells, the HGF protein was found to be expressed in a subset of leukemic cells in all patients tested. In addition, CML cells were found to express HGF mRNA as well as c-MET mRNA. HGF and c-MET transcript levels were found to vary from patient to patient without a clear correlation to the phase of disease. In consecutive experiments, we were able to show that basophils are the primary source of HGF in CML patients. In particular, highly enriched sorted CD203c$^+$ CML basophils were found to express HGF mRNA as well as the HGF protein. Correspondingly, HGF transcripts and the HGF protein were detectable in the basophil-committed CML cell line KU812, but not in K562 cells. c-MET mRNA was found to be expressed in highly enriched CD34$^+$/CD38$^-$ CML stem cells, and more mature CD34$^+$/CD38$^+$ CML progenitor cells, but less abundantly in mononuclear cells. We next asked whether expression of HGF in CML cells (basophils) depends on BCR/ABL kinase activity. For this purpose, Ba/F3 cells with doxycycline-inducible expression of BCR/ABL were employed. However, BCR/ABL failed to induce expression of HGF mRNA or the HGF protein in Ba/F3 cells. Correspondingly, the BCR/ABL-blocker imatinib, despite of its growth-inhibitory effects, failed to inhibit HGF expression in primary CML cells or in KU812 cells. Together, these data suggest that HGF is a BCR/ABL-independent basophil-derived mediator in CML. Basophils and basophil-derived mediators may play a more active role in disease biology and progression in CML than has so far been assumed. Whether basophil-derived HGF can act as an ´intraclone-paracrine´ regulator of CML stem cells is currently under investigation.

0798

AURORA KINASE EXPRESSION IN CHRONIC MYELOID LEUKAEMIA: POTENTIAL MARKERS OF DISEASE PROGRESSION

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Background. In Chronic Myeloid Leukaemia (CML), there is significant heterogeneity of disease behaviour and patient response to Imatinib, the front line therapy. Disease progression from chronic phase to blast crisis (BC) is accompanied by multiple additional genetic abnormalities. *Aims.* In this project, we targeted expression of genes that may be relevant to disease progression. Aurora Kinase A, B and C (AURKA, AURKB and AURKC, respectively) have important roles in regulating cell division. AURKA and AURKB over-expression is frequently observed in solid-tumour malignancies and has been associated with chromosomal instability (CIN) and aneuploidy. *Methods.* Gene expression of AURKA, AURKB, and AURKC was determined using quantitative real-time PCR in a total of 75 patient samples, in varying stages of CML, and 23 normal blood samples. Differences in expression between normal, diagnostic, BC and remission samples were evaluated using nonparametric ANOVA and Dunn's Post Test. *Results.* AURKA and AURKB were found to be significantly (P<0.001) over-expressed at presentation and at AP/BC compared with normal and remission levels. AURKC was significantly (P<0.001) under-expressed at presentation compared with expression of normals and at BC and remission. Further analysis of a subset of patients showed that expression levels of AURKB at diagnosis was higher in those who subsequently progressed to BC compared to those who had a sustained remission, at a level which approached significance (P=0.07). *Conclusions.* The expression levels of AURKA and AURKB are significantly elevated at diagnosis and at BC compared to normal and remission samples, whilst AURKC is significantly under-expressed at diagnosis. Higher expression of AURKB at diagnosis is associated with progression to BC, and may be useful as a prognostic indicator.

0799

GENE EXPRESSION PROFILE OF HEMATOPOIETIC PROGENITOR CELLS VS. GRANULOCYTES IN CHRONIC MYELOID LEUKEMIA

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Chronic myeloid leukemia (CML) is a malignant disorder of the hematopoietic stem cells characterized by the BCR-ABL oncogene. Previous microarray analysis of CML patients has been performed on CD34$^+$ hematopoietic progenitor cells and mononuclear cells. We expanded studies to combined analysis of CD34$^+$ cells and granulocytes to determine persistent and transient genes, related to various pathways, during cell maturation. Gene expression patterns reflect BCR-ABL-induced functional modifications such as increased cell-cycle and proteasome activity. The microarray analyses have been performed in 10 patients with CML and 8 healthy donors in their CD34$^+$ hematopoietic progenitor cells and granulocytes from peripheral blood. All observed CML Ph$^+$ patients were in chronic phase, with average of 1790 CD34$^+$ cells/μl in peripheral blood. The total gene expression in CML patients was 5248 and 3428 genes in CD34$^+$ cells and granulocytes, respectively what doubled the genes expression in corresponding cells of healthy donors as measured by microarray analysis. The 1620 genes had statistically significant difference (P<0.01) in expression between controls and CML, after 75% filtration, in CD34$^+$ hematopoietic progenitor cells. Restriction of the mean difference, with ±2.5, concentrated the statistical significance to 132 genes. The prominent genes with increased expression were NMI, MIF, HMGN2, HINT1, GCSH, SERBP1 and with decreased expression ADAMTSL3, GNGT1, SERPINB9, BNIP3L, MLLT4 in CD34$^+$ hematopoietic progenitor cells of CML patients. Among genes linked to inhibition of cellular proliferation by Gleevec: MYC and RAF1 had increased expression (2.5 fold), whereas STAT1 and FOS had decreased expression (1.8 fold) in CML. The following genes CDK4 and CDK6 had increased expression and NFKBIA, E2F1 and KRAS genes reduced expression in CML-linked genes of CD34$^+$ cells. The acceleration phase, of CML, sustained genes expression pattern comparable to chronic phase, as well as their ratio to control cells. Expanded microarray analysis of granulocytes revealed 57 statistically significant genes between CML and healthy donors. The following genes THRB, NAMPT, DBX1, TCF7, METRN continued the statistical significance in CML granulocytes *vs.* healthy donors, but with opposite difference in comparison to CD34$^+$ cells. The genes LIN7C, IL3F, REST and ST7L had increased expression in CML granulocytes, whereas TRNT1, SIGLEC1, TCF7, WNK3 and JUNB genes had reduced expression matched wih healthy donors granulocytes. Regarding cell cycle, the new genes emerged: CDC23, CDC26, CHEK1, ANAPC7, as totally absent in corresponding control CD34$^+$ hematopoietic progenitor cells. The cell cycle related genes TFDP1, CDC16, CDK6, SKP1, YWHAQ had elevated expression, in contrast to GADD45B, CDKN2B, CREBBP and E2F1 genes with reduced expression in CD34$^+$ cells of CML patients. This observation highlights the difference in genes expression between primitive and mature cells of CML patients, with the accent on hematopoietic progenitor cells which direct the pathogenetic course of malignancy. Presence of BCRABL fusion gene doubled the quantity of various genes included in regulation of cell cycle, cell

growth, proliferation and leukocyte homeostasis in hematopoietic progenitor cells. This tendency has been largely diminished, or even inverted, in granulocytes of CML patients.

0800

WT1 AND PRAME EXPRESSION IN CML PATIENTS PREDICT IMATINIB RESISTANCE AND BCR/ABL GENE MUTATIONS

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Background. WT1 and PRAME gene expression detection is broadly used for MRD monitoring of leukemia. Both those genes and their proteins are considered to be a perspective targets for immunotherapy approaches based on anti-tumor vaccination. It was shown that in CML patients there is a strict correlation between dynamic of BCR/ABL and WT1 expression. In our preliminary experiments it was found that CML patients treated by imatinib at the point of 12 mths have a different level of WT1 or PRAME gene expression. It is also know that not all CML patients have the same response on the imatinib therapy. It was shown (J Radich, PNAS 2006) that WT1 and PRAME are overexpressed in blastic crisis of CML. Tacking together these observations we have supposed that WT1 and PRAME gene activation may be connected with some of the basic features of CML progression. We have also assumed that resistance against the therapy of tyrosine kinase inhibitors (TKIs) may be reflected by changes in activity of those genes. Moreover, we have believed that BCR/ABL mutations that are common in cases of TKI resistance may occur in CML patients with some peculiar expression pattern of those genes and it reflects a period of genomic instability that tumor cells have inevitably to run through before obtaining BCR/ABL structure changes. We have decided to see if WT1 and PRAME may be an example of such genes. *Aim.* To study WT1 and PRAME expression levels in CML pts (N=51) at the point of 12 mths and then to see if it may influence future resistance. We have also intended to search for BCR/ABL mutations in resistant CML pts (N=132) and to compare this data with the WT1 and PRAME gene level. *Methods.* This study was based on RQ-PCR testing. We also use a direct PCR fragment sequencing to detect BCR/ABL mutation in imatinib resistant pts. *Results.* WT1 and PRAME expression level was detected in 51 CML pts. Using ELN criteria we register imatinib resistance or suboptimal response in 27 of 51 (54%) in this cohort of pts during the next 6-24 mths of treatment. In 27 resistant pts 21 (78%) had WT1/ABL expression at 12 mths > or = 0,001%. In 24 good responders 16 (67%) had WT1/ABL expression at 12 mths < 0,001%. Coefficient of asymmetry in this case was 7,5 (much more then 1), P=0,000. There was no significant difference between good and bad responders according PRAME expression. Nevertheless, among 132 resistant CML pts in 62 (47%) of them that had BCR/ABL mutations 59 (80%) showed PRAME/ABL meaning > or = 0,1%. In 70 of 132 (53%) BCR/ABL mutation negative pts 58 (83%) have PRAME level <0,1%. Coefficient of asymmetry was 18, P=0,000. *Conclusion.* In the case of CML high WT1 expression is a predictor of imatinib resistance and high PRAME expression in resistant pts is a predictor of BCR/ABL mutations.

0801

HIGH RESOLUTION MELT CURVE ANALYSIS AS A TOOL FOR SCREENING TYROSINE KINASE DOMAIN MUTATIONS IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA ON TYROSINE KINASE INHIBITORS

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Background. Imatinib mesylate (IM), a tyrosine kinase inhibitor (TKI) currently remains the treatment of choice for patients with chronic myeloid leukemia (CML). Continuous administration of IM is however often complicated by the development of clinical resistance and subsequent loss of cytogenetic or molecular remission. One of the most prevalent mechanisms of resistance is through the acquisition of point mutations within the ABL kinase domain of the BCR-ABL fusion gene. Screening and identification of these TKD mutations are thus important in the management of patients with CML on TKIs. Objective. The routine identification of TKD mutations involving direct nucleotide sequencing has a detection sensitivity of about 20% and is generally not suitable for high-throughput screening. We sought to identify if high resolution melt (HRM) curve analysis could instead be used as an initial screening tool for TKD mutations. *Methods.* RNA was extracted from 94 archived serial samples of 45 patients in chronic or accelerated phase and was subjected to semi-nested RT-PCR targeting the TKD of the ABL gene, followed by direct-sequencing of the second step PCR product. The BCR-ABL gene of the same set of samples were in parallel, selectively amplified by conventional RT-PCR, followed by HRM assay using four pairs of primers targeting four overlapping regions that span the entire ABL kinase domain. Amplicons with a shift in HRM differential curves were purified and subjected to direct sequencing to confirm the mutation. Detection sensitivity of the HRM assay was assessed by diluting plasmids containing the specific mutation in wild type DNA from 5% to 100%. *Results.* Twenty of the 94 samples were not evaluable due to poor amplification quality. Twenty-two mutations were detected in 15 samples by direct sequencing. The mutation types detected in these samples included M244V, L248V, G250E, Y253F, T315I, A341T, F359V and E459Q. HRM successfully recognized the presence of 20 of the mutations. The failure of detecting one of the mutations was attributed to poor PCR amplification prior to HRM caused by RNA degradation while one of the samples harbouring G250E did not show a temperature shift due to the presence of 100% mutants as compared to wild type and absence of heteroduplexes. However, HRM detected the additional presence of Y253F mutation in a sample with multiple mutant types that had not been detected by nucleotide sequencing. Five samples that indicated presence of mutants due to temperature shifts were confirmed as wild type by nucleotide sequencing. Plasmid dilutions showed that HRM was capable of detecting 5% of mutant types in wild type DNA. *Conclusion.* The high sensitivity, rapid throughput and cost-effectiveness of HRM make it an attractive screening tool for early identification of CML patients on TKIs who may harbour TKD mutations. Wild type DNA-spiking is however essential in 100% mutant samples in order to form heteroduplexes for better melt curve discrimination. False positive results are also liable to occur with a test specificity of 92%. Samples screened positive by HRM should be subjected to nucleotide sequencing for confirmation.

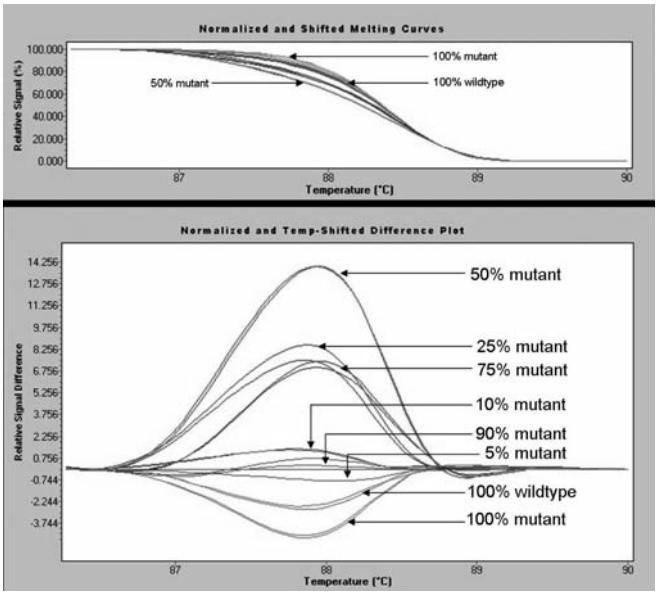

Figure 1. Sensitivity detection of HRM assay.

0802

THE ROLES OF CERAMIDE GENERATING AND CERAMIDE CLEARENCE GENES IN APOPTOSIS IN CHRONIC MYELOID LEUKEMIA CELLS EXPOSED TO DASATINIB

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Backround. Chronic myeloid leukemia (CML) is a hematopoietic stem cell disorder that results from the reciprocal translocation of chromosomes 9 and 22. As a result of this translocation, the oncoprotein BCR/ABL is generated. (1) Dasatinib is a rationally designed, multitargeted kinase inhibitor that inhibits the tyrosine kinase activity of this

oncoprotein. (2) Ceramide is a molecule that plays a vital role in lipid metabolism. Ceramide is a novel regulator of differentiation, senescence, proliferation, cell cycle and also acts a strong apoptotic molecule.(3) In this study, we investigated the possible synergistic apoptotic effects of intracellular ceramide concentrations and dasatinib on human K562 and Meg-01 chronic myeloid leukemia (CML) cells, and changes in expression levels of ceramide metabolizing genes in response to dasatinib. *Methods.* The cytotoxicity analyses of dasatinib, C:8 ceramide to induce *de novo* generation of ceramide, PDMP to inhibit glucosyl ceramide synthase (GCS) and SK1 inhibitor to inhibit the conversion of ceramide to antiapoptotic sphingosine-1-phosphate(S1P) were carried out by XTT cell proliferation assay. The changes in caspase-3 enzyme activity and mitochondrial membrane potential (MMP) were measured by caspase-3 colorimetric assay and JC-1 MMP detection kit, respectively. *Results.* We have shown that dasatinib induces apoptosis and arrest the cell-cycle for both cells in a dose dependent manner. When we treat the cells with C:8 ceramide in addition to dasatinib application we have observed significant induction of apoptosis. On the other hand, inhibition of conversion of apoptotic ceramides to anti-apoptotic glucosyleceramide or to sphingosine-1-phophate increased cytotoxic effects of dasatinib as compared to any agent alone. These results are proved not only by decreases in cell proliferation, but also by increases in cytoplasmic/monomeric JC-1 and caspase-3 enzyme activities. RT-PCR results revealed that there were downregulations in expression levels of antiapoptotic GCS and SK-1 genes. On the other hand, while there were increases in expression levels of LASS1, -2, -4, -5, and -6 in K562 cells, in contrast to our expectations, there were downregulation in expression levels of LASS genes in Meg-01 cells in response to increasing concentrations of dasatinib. Conclusion. Taking together all these results have shown for the first time that targeting ceramide metabolism in addition to inhibition of BCR/ABL by dasatinib induces apoptosis synergistically in Philadelphia positive CML cells.

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0803

LEPTIN PRODUCTION AND LIPID RELEASE BY BONE MARROW ADIPOCYTES REGULATE IN VITRO THE EVOLUTION OF A MYELOID LEUKEMIC CELL LINE

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Background. We have recently demonstrated that bone marrow (BM) adipocytes inhibit granulocyte differentiation by a mechanism implicating cell-cell contact and leptin production (Belaid Z *et al.*, Stem Cells 2008). They were also described *in vivo* as negative regulators of the hematopoietic microenvironment (Nature, 2009, 460:259). Aim of the work. We postulate that BM adipocytes could also influence malignant myeloid hematopoietic cells. Materials and *Methods.* We realized co-cultures of a leukemic myeloid precursor cell line (LC) exhibiting characteristics of LMC with BM adipocytes or with a BM stromal cell line (HS-5) as control. LC were also cultured in fat medium and/or leptin. Culture medium was analysed by proteome array and ELISA. The phenotype of LC was tested by immunostaining and FACS analysis. BCL-2 expression and NFKB pathway were tested by western blot. *Results.* BM adipocytes, but not HS-5, produce high levels of leptin (100pg/mL) and induce accumulation of lipids in leukemic cells (LC). They increase OB-R (leptin receptor) and TLR-4 expression in LC, activate NFKB pathway, decrease BCL-2 expression and partially inhibit LC proliferation. Lipids added to the culture medium of LC exert the same effects except that they induce an increase in BCL-2 expression. Leptin alone does not have any influence on LC but restores the decrease in the anti-apoptotic BCL-2 expression observed in the presence of BM adipocytes. Adipocytes seems thus to negatively regulate leukemic cells. However, adipocytes, but not HS-5, display morphological evidence of cell death after one week in co-culture with LC: the presence of pro-inflammatory cytokines produced in the culture medium as a consequence of the activation of NFKB pathway induced by the binding of fatty acids to TLR-4 could explain this effect. *Conclusion.* We think that BM adipocytes could negatively regulate myeloid malignant cells in the first stage of the disease; their disappearance in leukemic BM in response to pro-inflammatory cytokines could favour the evolution of the disease.

0804

P-CRKL IS NOT A SURROGATE OF BCR-ABL ACTIVITY *IN VITRO* AT SHORT TIME-POINTS

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Background. Phosphorylation of Crk-like (CrkL) protein, a downstream Bcr-Abl binding partner, is commonly used as a surrogate marker for Bcr-Abl kinase activity and response to tyrosine kinase inhibitor (TKI) treatment in Chronic Myeloid Leukemia (CML). However, the accuracy of phospho-CrkL (p-CrkL) as a reflection of Bcr-Abl status at early time-points during *in vitro* TKI treatment (<24h) has not been examined. *Aims.* To investigate if p-CrkL is an accurate surrogate marker of Bcr-Abl kinase activity after TKI treatment in K562 CML cells at early time-points. *Methods.* In this study, K562 CML cells were treated with imatinib or dasatinib. At each time-point, cells were collected and lysed with NP-40 lysis buffer. P-CrkL and tyrosine phosphorylated Bcr-Abl (p-Tyr Bcr-Abl) protein levels were measured by Western blotting, and densitometry was performed. Values are shown as percentage to no drug control. *Results.* After 1μM imatinib treatment, p-Tyr Bcr-Abl level dropped quickly to 24.9%±7.7% of no drug control by 1 h and then plateaued at this level to 48 h, while p-CrkL level dropped to 59.3±13.5% at 0.5 h, but then recovered to 94.4±17.3% at 4 h (P=0.012), before dropping again to 39.0±13.4% at 48 h. The difference between these responses of p-CrkL and p-Tyr Bcr-Abl is statistically significant (P=0.023 at 4 h; P=0.008 at 8 h; P=0.0009 at 24 h) (Figure 1). Furthermore, as p-Tyr Bcr-Abl level dropped sharply within 1 h, we looked at earlier time-points from 1 minute of imatinib treatment. We observed a rapid reduction in p-Tyr Bcr-Abl to less than 50% of no drug control by 10 minutes, however p-CrkL remained at control levels during this period. The difference between p-CrkL and p-Tyr Bcr-Abl is statistically significant (P<0.05 at 5 and 10 minutes; P<0.01 at 15, 30 and 60 minutes). With 10 nM dasatinib, the p-Tyr Bcr-Abl response was similar to that seen with imatinib - dropping rapidly to 14.3±2.0% at 1 h then remaining around this level. However, unlike imatinib, dasatinib caused a strong reduction in p-CrkL to 18.8±4.7% by 1 h, and this reduction was then sustained with no recovery of phosphorylation. We hypothesized the initial sharp drop of p-Tyr Bcr-Abl level after TKI treatment (within 1 h) may be due to protein tyrosine phosphatase (PTPase) driven active dephosphorylation. Therefore, we treated K562 cells with both imatinib and PTPase inhibitor, sodium stibogluconate (SSG). SSG partially inhibited the initial drop of p-Tyr Bcr-Abl level caused by imatinib, suggesting PTPases play a role in Bcr-Abl dephosphorylation. Summary: These results demonstrate that p-CrkL is not a reliable indicator of Bcr-Abl kinase activity within 24 h of imatinib treatment and indicate that the early responses to imatinib and dasatinib differ. Importantly, these data also show that in addition to inhibition of the Bcr-Abl kinase activity there is a rapid and active dephosphorylation of Bcr-Abl within 1 h of TKI treatment, driven at least in part, by PTPase activity.

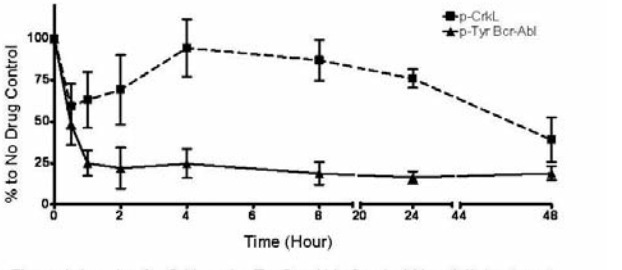

Figure 1: Levels of p-CrkL and p-Tyr Bcr-Abl after 1 μM imatinib treatment measured by Western blotting. Data are shown as percentage to no drug control (mean ± SEM, n=3).

Figure 1.

Chronic myeloid leukemia - Clinical 2

0805

SECOND MALIGNANCIES IN 559 PATIENTS WITH CHRONIC MYELOID LEUKEMIA TREATED WITH IMATINIB FRONTLINE: DATA FROM THE GIMEMA CML WORKING PARTY

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Background. Imatinib, a TKI inhibitor that revolutionized CML therapy, is now approaching ten years since its commercialization. Until now, even in complete durable molecular response, discontinuation of imatinib is not recommended, and imatinib remains a life-saving drug to be taken chronically. For this reason an important issue is represented by long-term side effects and among these the incidence of second malignancies. Roy *et al.* (Leukemia 2005) reported an unexpected incidence of second neoplasms in patients treated with imatinib after interferon (6/189 patients, 3.2%; urinary tract cancer: 4/6); in contrast, an analysis performed by Novartis in 9518 patients treated with imatinib all over the world did not provided evidence for an increased overall incidence of second malignancies (Leukemia 2006). However few data are available from independent studies, and particularly in imatinib-only treated patients. *Aims.* to evaluate the incidence of second malignancies in patients treated with imatinib frontline, enrolled in 3 multicentre independent trials. *Methods.* overall, 559 patients have been enrolled in 3 concurrent clinical studies of the GIMEMA CML Working Party: CML/021, Imatinib 800 mg in intermediate Sokal risk patients (Clin Trials Gov. NCT00514488); CML/022, Imatinib 400 mg *vs.* 800 mg in high Sokal risk patients (Clin Trials Gov. NCT00510926); CML/023, observational, Imatinib 400 mg. The median age was 52 (extr.18 - 84) years; 308 patients (55%) were ≥50 years; the median follow-up is currently 54 months. *Results.* during the follow-up, 14 patients (2.5%) developed a second malignancy at a median distance of 16.5 months (range 2 - 52) from the start of imatinib therapy (Table 1); 4 of these tumors (2 colon cancer and 2 NHL) were diagnosed within 6 months. All patients were older than 50 years (median 64, extr. 53 - 77) at the diagnosis of the second malignancy. Eleven out of the 559 (2%) patients died due to second neoplasm progression. According to epidemiologic data (Registro Tumori) in Italy, the annual incidence of neoplasm varies from 1% in the range of age between 50 and 69 years and 3% for patients over 70 years. *Conclusion.* in this population of CML patients imatinib-treated, the incidence of second neoplasm do not differ from the observed incidence of neoplasm in the national population. In particular, in contrast to what previously reported, no increased incidence of urinary tract cancer was observed.

Table 1.

Patients	Diagnosis of CML	Age	Second Neoplasm (2nd Neop.)	Diagnosis of 2nd Neop.	Distance CML–2nd Neop. (months)	Status of CML	Alive
1	10/2004	69	Billar Duct	11/2007	37	CCyR/MMR	No
2	10/2004	50	Breast	02/2009	52	CCyR/MMR	Yes
3	10/2005	76	Breast	05/2007	18	PCyR	No
4	09/2004	61	CNS	06/2007	33	CCyR/MMR	No
5	10/2005	60	CNS	12/2007	26	CCyR/MMR	No
6	05/2004	53	Colon	07/2004	2	n.a.	No
7	05/2005	63	Colon	03/2007	22	CCyR/MMR	No
8	03/2006	60	Colon	05/2006	2	n.a.	No
9	11/2004	56	Oesophagus	10/2006	23	CCyR/MMR	No
10	10/2005	74	Kidney	01/2007	15	CCyR/MMR	Yes
11	03/2005	64	NHL	09/2005	6	CCyR/MMR	No
12	04/2005	77	NHL	04/2006	12	CCyR/MMR	Yes
13	03/2005	64	NHL	07/2005	4	CCyR/MMR	No
14	06/2005	70	Pancreas	05/2006	11	CCyR/MMR	No

CNS: central nervous system; NHL: non-Hodgkin lymphoma ; CCyR : complete cytogenetic response ; PCyR : partial cytogenetic response ; MMR : major molecular response ; n.a.: not available

0806

CHRONIC MYELOID LEUKEMIA (CML) PATIENTS WITH E14A2 BCR-ABL FUSION TRANSCRIPT ACHIEVE A FASTER MAJOR MOLECULAR REMISSION (MMR) ON IMATINIB THAN THOSE EXPRESSING THE E13A2 TRANSCRIPT

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Background. BCR-ABL positive CML in the vast majority of cases is associated with the expression of one or both of e13a2 and e14a2 BCR-ABL fusion transcripts. Optimal response to imatinib treatment of CML is defined by the achievement of an MMR after 18 months, i.e. the reduction of the BCR-ABL expression below 0.1% according to the international scale (IS). Several markers have been evaluated to predict treatment response, however few data have been presented concerning the impact of the BCR-ABL transcript type in the imatinib era yet. *Aims.* We have evaluated the prognostic impact of the BCR-ABL transcript type on long term molecular and cytogenetic outcome in CML patients on first line imatinib based treatment. *Methods.* 1,036 patients (pts) with newly diagnosed CML (61% male, median age 53 years, range 16-86) were included into the randomized German CML Study IV and treated with an imatinib based therapy consisting of standard dose imatinib (400 mg/d), high dose imatinib (800 mg/d) and combinations of standard dose imatinib with low dose cytarabine or interferon alpha. BCR-ABL expression (IS) was determined by quantitative RT-PCR from 9,072 peripheral blood samples with a median follow up of 33 months (range 0-88). The type of BCR-ABL transcript was defined by multiplex PCR. Cytogenetic response of 949 patients was determined by conventional metaphase analyses of 4,050 bone marrow samples with a median follow up of 21 months (range 0-84). *Results.* Multiplex PCR identified three groups of patients: (i) e13a2 transcript, n=424; (ii) e14a2 transcript, n=464; (iii) e13a2 and e14a2 transcript, n=148. The median time to achieve MMR was 17.5 months (i), 13.4 months (ii) and 13.5 months (iii) revealing a significant difference between group (i) and (ii) (P=0.0072). Group (iii) did not differ significantly from groups (i) and (ii) (P=0.09, P=0.79). Cumulative incidence of achieving MMR was 66.3% for group (i), 73.9% for group (ii) and 72.3% for group (iii). The analysis of time to achieve a complete cytogenetic remission (CCyR) showed a median of 9.4 months (i), 9.0 months (ii) and 9.2 months (iii) indicating no significant differences between groups. 73.2% of pts achieved CCyR in group (i), 75.2% in group (ii) and 73.1% in group (iii). *Conclusions.* The type of BCR-ABL transcript is predictive for the achievement of major molecular response (MMR). Median time to MMR for patients expressing e14a2 is about four months shorter than for patients with e13a2. Regarding the cytogenetic level of response no significant impact of BCR-ABL transcript type could be revealed.

0807

REAL WORLD DATA ON CHRONIC MYELOID LEUKEMIA - A REPORT FROM THE SWEDISH POPULATION BASED CML-REGISTRY

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Background. The Swedish CML Registry was founded in 2002 by the Swedish CML group and the Swedish Society of Hematology. It is supported by the Swedish Board for Health and Welfare, and run in collaboration with the Oncological Centres in each of the six Swedish health care regions, covering in total a population of 9 million. Each region contains one or two university hospitals and up to twelve county hospitals treating patients (pts) with CML. Clinicians are supposed to report all pts with newly diagnosed CML to this registry. In addition, pathologists and clinicians are obliged by law to report all new cases to the Swedish Cancer Registry. *Aims.* To establish patient characteristics and treatment outcome in a population-based cohort of CML patients after the introduction of imatinib. *Methods.* Pts >16 yrs with CML diagnosed Jan. 1

2002 or later are included. Pts with clinicopathological findings typical for CML but cytogenetics not performed are also accepted. At diagnosis, disease stage, CBC, Sokal score, WHO PS, cytogenetics and aim of treatment are registered. At an annual follow-up, principal treatment and response are reported. Date-of-death data are collected directly from the Swedish Population Registry. *Results.* During 2002-2006, 423 (m/f: 212/211) new cases of CML were reported from 55 hospitals, covering 98 % of all CML cases diagnosed in Sweden according to the compulsory Swedish Cancer Registry. This corresponds to an annual CML incidence of 0.9/100 000. Median age was 58 yrs (17-99), out of which 25% were ≥70 yrs. Diagnosis was confirmed by karyotyping in 82%, FISH (+/-RT-PCR) 7%, RT-PCR only 7%, whereas cytogenetics was not performed or unsuccessful in 4% (n=19; median age 80 yrs). Three hundred-eighty-eight pts (92%) were diagnosed in CP, 14 (3%) in AP, 10 (2%) in BC, data missing in 10 cases (2%). Data on Sokal score was available in 85% of pts with CP: HR 36%, IR 41% and LR 23%. Treatment aim was "CCgR"in 81%, and "palliation only"in 19%. Only 59 pts (14%) were included in an upfront study protocol according to the reporting clinician. Imatinib was "initial treatment"in 75% and 41% of pts <70 and ≥70 yrs, respectively (proportion of imatinib treated pts increasing during the study period). As of Febr.17 2010 (median follow-up: 62 months), 338/423 pts (80%) are alive. In pts with CP at diagnosis, the projected 4-yrs survival for Sokal-LR/IR, Sokal-HR- and Sokal-missing patients, is 88.7% (84.4-93.2), 76.0% (68.6-84.2) and 73.6% (63.0-86.0), respectively (95% CI). Updated results will be presented at the meeting. *Conclusions.* Establishing a population-based national CML registry is feasible. Only a minority of CML patients are included in upfront clinical studies. A relatively large proportion of elderly pts diagnosed during this time period (2002-06) received palliative treatment with mainly non-imatinib regimens.

0808

THE PRE-TREATMENT LEVELS OF PROTEIN PHOSPHATASE 2A (PP2A) AND ITS INHIBITOR SET CAN PREDICT PATIENTS DESTINED TO PROGRESS TO BLAST CRISIS OF CML

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Background. BCR-ABL tyrosine kinase activity induces and maintains chronic myeloid leukaemia, but the molecular factors that contribute to disease progression are not well understood. PP2A is a phosphatase which regulates cell proliferation, differentiation and survival. Blast crisis of CML has been associated with increased levels of SET, a key inhibitor of PP2A function. (Nevaiani *et al.*, Cancer cell 2005, Perrotti *et al.*, Br J Cancer 2006) However, only limited number of cases were reported and so the broader implications of PP2A in CML remain unclear. (Nevaiani *et al.*, Cancer cell 2005). *Aims.* The aim of this study was to investigate if PP2A or its inhibitory protein SET could predict clinical outcome in imatinib treated patients. *Methods.* We investigated the levels of PP2A, phosphorylated PP2A and its inhibitor SET in mononuclear cells from chronic phase CML patients by flow cytometry. Samples were taken at diagnosis and following 12 months of imatinib treatment (n=30). Patients were categorised into three groups - those who subsequently achieved a complete cytogenetic response (CCR group), those who did not achieve CCR though did not progress to advanced disease (no CCR group) and those who subsequently progressed to blast crisis (BC group). Results. At diagnosis, the CCR group had low levels of PP2A (compared to normals P=0.005), low PP2A activity (as indicated by high levels of tyrosine 307 phosphorylation) and high levels of SET protein, indicating impaired PP2A function. In the no CCR group the level of PP2A was significantly lower than in normal blood (P=0.005) and its activity was the lowest of all three patient groups;n SET level was also high. In sharp contrast, patients at diagnosis who were destined to later progress into advance disease were found to have significantly higher levels of PP2A (P=0.0001). The degree of PP2A phosphorylation was higher (low activity) than normals and SET levels were virtually undetectable (P=0.011 and P=0.035 compared to CCR and no CCR groups respectively). Following 12 months of imatinib treatment in the CCR group the levels of PP2A increased, its activity increased (reduced Y307 phosphorylation) and the levels of SET were decreased - all leading to an environment permitting achievement of CCRe. In the no CCR group PP2A levels increased and the amount of phosphorylated PP2A decreased -suggesting that the activity of PP2A had increased - but the function of PP2A remained impaired as the level of SET was not diminished. In the BC group the PP2A protein levels decreased and the activity decreased further. SET levels remained unchanged suggesting an alternative signalling pathway controlling PP2A function in this group. *Summary/Conclusions.* These data suggest that the levels of PP2A, phosphorylated PP2A and SET at diagnosis can give important prognostic information and may identify patients at high risk of disease progression for who strategies other than imatinib may be appropriate.

0809

IMATINIB TROUGH PLASMA LEVELS IN PHILADELPHIA POSITIVE CHRONIC MYELOID LEUKEMIA (CML) PATIENTS: RESULTS OF A MULTICENTER STUDY CSTI571AIL11TGLIVEC

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Background. Imatinib is the standard of care in the treatment of CML. Almost uniquely in the treatment of leukemia, pts are receiving a fixed dose regimen ranging between 400-800 mg/day. Imatinib trough plasma levels may vary among pts and may correlate with both response and toxicity. *Aims.* Moreover Imatinib plasma levels may predict response and thus serve as a guide in optimizing the anti leukemic effect. *Methods.* We evaluated Imatinib trough plasma levels, using liquid chromatography/mass spectrometry, in 166 CML pts (M-89; F-77), median age 55y (range, 18-85). Seven centers participated in the study, mostly referral university hospitals. None of the evaluated pts were part of an Imatinib clinical study. Pts had to receive Imatinib for at least one month prior to the single plasma level assessment. Pts compliance was assessed by diary logs. *Results.* All pts (96%; na-4%) were in CP. Disease duration was 3.6y (range 0.12-17.9). 76 % of the pts received Imatinib at the conventional dose of 400 mg/day, while 15 % of the pts received higher dose (600-800 mg/day). In 8% of the pts the Imatinib daily dose had to be reduced to 100-300 mg/day due to side effects. Compliance was 97%. Conventional cytogenetics was available in 89% of pts and FISH in 67%. 95% of the pts were positive for bcr/abl by PCR (na-5%). Abnormal liver and renal function tests were observed in 7.0% and 7.5% of the pts, respectively. Initial/previous therapies included: Hydroxyurea - 45.8%, Interferon alpha - 22.9% and Busulfan in 1.2% of the pts. 98.6% of the pts achieved CHR. 72% of the pts achieved stable CCyR and 4.8% had partial CyR, while 3.4% achieved only minimal CyR (mCyR) and 5.5% of pts had no CyR. 76% of the pts had MMolR, while 18.5% had minor MolR (mmolR). Imatinib plasma levels are available for 146 (88%) of the pts (20 pts-pending). Mean plasma levels was 1163+65 ng/mL. Median levels were 1001 ng/mL (range, 175-7460). Imatinib plasma levels were not different between pts achieving complete or partial CyR (1018+62 *vs.* 1077+125 ng/mL, respectively). While in pts that achieved only mCyR imatinib levels were significantly lower (621+ 138 ng/mL, P=0.03). Similarly, in pts that did not achieve CyR plasma levels were significantly lower 688 + 98 ng/mL, P=0.018. No difference was observed in Imatinib plasma levels between pts that achieved major *vs.* minor MolR, 1018+81 *vs.* 973+104 ng/mL, respectively. Abnormal liver or renal function did not affect median Imatinib plasma levels: 990 + 232 and 994 + 172 ng/mL, respectively. *Conclusions.* Imatinib trough plasma levels of 1163+63 ng/mL correlates with CCyR. A significant lower plasma level was observed in CML pts that failed to achieve or achieved only minor CyR. In the current assessment, plasma levels were determined in CP CML pts treated with Imatinib out of Imatinib clinical studies in 7 participating centers. It is thus possible to assess Imatinib plasma levels in common daily practice. Imatinib plasma levels may be a practical tool in optimizing Imatinib therapy for CML pts.

0810

A EUROPEAN OBSERVATIONAL STUDY OF DASATINIB IN THE MANAGEMENT OF IMATINIB-RESISTANT AND -INTOLERANT PATIENTS WITH CHRONIC MYELOID LEUKEMIA: FORTE STUDY (CA180-211)

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Background. Even though imatinib has transformed the outcome of patients diagnosed with chronic-phase (CP) chronic myeloid leukemia (CML), at 6-8 years follow-up, 45-56% of patients ultimately discontinue imatinib (Deininger *et al*. Blood 2009; Hochhaus *et al*. Blood 2008), mostly due to unsatisfactory response and/or toxicity. Clinical studies in imatinib-resistant patients with CP-CML suggest that earlier and deeper responses to second-generation TKIs (2G-TKIs) including dasatinib, a potent BCR-ABL kinase inhibitor, correlate with improved outcomes and that time from first detection of imatinib failure to beginning of 2G-TKI therapy is a significant predictor of response (Milojkovic *et al*. Haematologica 2009; Quintas-Cardama *et al*. Cancer 2009). However, little data has been gathered from real-life observation. *Aims.* FORTE (Factors impacting On Response To dasatinib in Europe) aims at identifying explanatory factors of response to dasatinib in a real-life observational, non-interventional study. This study investigates the relationship between time elapsed from detection of imatinib resistance/intolerance to initiation of dasatinib and best response to dasatinib (primary objective). Disease and treatment-pattern characteristics of the observed population monitored and treated with dasatinib in real life are described, and best response to dasatinib is summarized. *Methods.* This large international study, conducted in 141 sites across 12 European countries, enrolled imatinib-resistant/-intolerant CP-CML patients being treated with dasatinib for ≥2 months. Patients gave written informed consent before study entry. Best response to dasatinib was assessed by an ordered categorical variable which combines all types of responses achieved by patients and selects the highest level of response. Disease history, response to imatinib/dasatinib, response-monitoring rates and criteria to define imatinib resistance/intolerance, as determined by physicians' medical judgment, were collected from individual patient charts. The study is compliant with international guidelines. *Results.* This is the first preliminary analysis of 225 CP-CML patients (male/female:102/123). Median age was 56.6 years at start of dasatinib and median time from diagnosis to dasatinib initiation was 46.1 months. Intermediate/high Sokal and Hasford scores at diagnosis were seen in 74.6% and 78.6% of assessable patients, respectively. Imatinib resistance occurred in 133 patients, 62 were imatinib-intolerant and 30 were both imatinib-resistant and -intolerant. Overall, 54.7% of 223 evaluable patients achieved complete cytogenetic response (CCyR) or major molecular response (MMolR) as best levels of response with imatinib. Imatinib had been administered at standard dose (400mg/d) in 38.2% of patients, at 600 mg/d in 35% and at >600 mg/d in 26.7% of patients. Dasatinib was initiated at a median of 7.7 months after imatinib resistance/intolerance, at a starting dose of 100 mg/day in 70.5% of patients. Within the first 12 months of dasatinib therapy, 63.2% of the 215 evaluable patients achieved CCyR or MMolR. At this early assessment, there is yet insufficient evidence to conclude on the primary objective analysis. *Summary/Conclusions.* To date, FORTE data are consistent with results from investigational Phase II and III clinical trials, confirming dasatinib effectiveness in imatinib-resistant/-intolerant patients in a real-life observational study. Updated results, including those related to the study primary objective, will be presented at the congress.

0811

OCCURRENCE OF PLEURAL EFFUSIONS DURING DASATINIB TREATMENT IN ELDERLY CML PATIENTS

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Background. Pleural effusions are the most common extra-haematological toxicity observed during Dasatinib (DAS) treatment: their occurrence often requires treatment discontinuation and impairs the otherwise high efficacy of the drug. Aims and Methods We revised 125 patients with Chronic Myelogenous Leukemia (CML) in chronic phase treated in 21 Italian Centers, who received DAS when aged >60 years after being resistant/intolerant to Imatinib (IM), in order to find the most important prognostic factors for pleural effusions. Results There were 63 males and 62 females, Sokal risk score at diagnosis was low in 32 patients, intermediate in 49, high in 19 and not evaluable in 23; median age at DAS start was 69.9 years (IR 65.4-74.4), median interval from diagnosis to DAS therapy was 75.8 months (IR 39.1 - 115.9), 57/125 patients (45.6%) had received an IFN-based therapy before IM. As to IM treatment, all but 9 patients had initially received the 400 mg daily standard dosage and 58/125 patients (46.4%) had increased the dosage to 600 - 800 mg/day; on the whole, median period of IM treatment was 46.6 months (IR 21.8 - 61.8). Starting dosage of DAS was 140 mg in 52 patients, 100 mg in 56 patients and <100 mg in the remaining 17 patients. During treatment, 41/125 patients (32.8%) presented a pleural effusion, which was grade 2 in 31 patients and grade 3 - 4 in 10 patients (24.4% of the 41 patients with pleural effusion and 8% of the entire cohort of patients), according to WHO scale; in 11/41 patients (26.8%), there was a concomitant pericardial effusion. In addition, pleural effusion was recurrent in 19/41 patients (46.4%). Median time from DAS initiation to occurrence of 1st pleural effusion was 10.4 months (IR 2.2-18.1). Among 41 patients with pleural effusion, 8 (19.5%) did not require DAS interruption while 33 (80.5%) discontinued the drug; however, DAS was successfully resumed in 28/33 patients (84.8%) after a median period of 20 days (IR 15 - 38) and, overall, only 5 patients (12.1% of the 41 patients with pleural effusion and 4% of the entire cohort of patients) required permanent DAS discontinuation. The predictive role for pleural effusions of several characteristics (sex, age, Sokal risk, smoke attitude, concomitant cardiological or pulmonary diseases, concomitant diuretic treatment, interval from diagnosis to DAS, state of CHR at DAS start, DAS initial dosage) was evaluated in univariate analysis; only presence of concomitant pulmonary disease (P=0.002), initial dose of DAS (140 mg *vs.* 100 mg, P=0.01) and Sokal risk at diagnosis (high *vs.* low-intermediate, P=0.043) were significant. There were no differences among patients with or without pleural effusions as concerns response rates and overall survival. Conclusions. Pleural effusions were common in our unselected population of elderly patients but were clinically manageable and did not seem to affect treatment results; however, a complete pulmonary evaluation before treatment could be useful to tailor the treatment with DAS in elderly patients.

0812

NILOTINIB 300 MG TWICE DAILY IS EFFECTIVE AND WELL TOLERATED AS FIRST LINE TREATMENT OF PH-POSITIVE CHRONIC MYELOID LEUKEMIA IN CHRONIC PHASE: UPDATED RESULTS OF THE ICORG 0802 PHASE 2 STUDY

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Background. Imatinib (IM) 400 mg daily is currently the standard treatment for early chronic phase (ECP) CML. Preliminary results of the recent ENESTnd phase III trial suggests that Nilotinib, a more potent, second generation Abl inhibitor may be superior to imatinib as initial treatment of ECP CML at doses of both 300 mg and 400mg BID. *Aims.* We wished to explore the lower dose schedule, 300 mg BID, believing this could be as effective as 400 mg BID but with the potential for fewer adverse events, as well as fewer interruptions and dose reductions which may be important in achieving deep molecular responses. *Methods.* Since December 2008, ICORG, the All-Ireland Cooperative Oncology Research Group has been conducting an open-label, single stage, multicenter, phase II study (ClinicalTrials.gov NCT00809211) to investigate the safety and efficacy of nilotinib 300 mg BID in untreated, ECP, Ph-pos CML patients. The primary endpoint is the CCyR rate at 6 months; secondary endpoints include the kinetics of molecular response, determined by Q-PCR at baseline and 3 monthly from start of treatment. *Results.* To date a total of 27 patients have been enrolled. This abstract is limited to the 18 patients evaluable for response after at least 3 months follow up. The median age of these 18 patients is 51 (range 20-77); 50% have low risk Sokal score, 11% intermediate and 39% high risk. Median follow up is currently 8 months (range 4-14). By intent to treat analysis at 3 months the CHR rate is 100%, the CCyR rate is 56% and MMR rate is 28%. Following 6 months 14/15 patients have achieved CCyR (100%) while 10/14 patients have achieved MMR (71%). While none of the 18 patients have progressed on study, 2 patients have undergone dose escalation to 400mg BID for suboptimal response. The median daily dose was 600mg (range 218-775mg n=18); 12/18 (67%) interrupted nilotinib at least once with a median duration of interruption of 7 days. The dose of nilotinib at the last visit was 400 mg BID in 2/18 patients (11%), 300mg BID in 14/18 patients (79%), 200 mg BID in 1/18 patients (5.6%) and 200mg OD in one patient (5.6%). Haematologic toxicity was minimal with a single episode of transient grade III thrombocytopenia (5.6%). Grade III non-haematologic toxicity included an elevated lipase in 4/18 (22%). The only other grade III toxicities noted were musculo-sleketal pain and an elevated ALT in 1 patient each. There were no grade IV toxicities. *Conclusions.* Nilotinib 300 mg BID induces high rates of CCyR and MMR within 6 months, equivalent to those reported thus far in phase II and III studies of nilotinib in ECP CML. These responses have been achieved with minimal toxicity and dose intensity has been well maintained with most patients taking at least 300 mg BID at last follow up. The early results of this trial provide independent confirmation that nilotinib 300 mg BID is safe and effective treatment for ECP CML.

0813

IDENTIFICATION OF UNBALANCED GENOMIC ABERRATIONS BY ARRAY CGH IN CHRONIC MYELOID LEUKEMIA PATIENTS WITH SUBOPTIMAL RESPONSE TO IMATINIB TREATMENT

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Background. Chronic myeloid leukemia (CML) is associated with the presence of the Philadelphia chromosome (Ph), t(9;22)(q34;q11), leading to the expression of the aberrant tyrosine kinase fusion protein, BCR-ABL. The tyrosine kinase inhibitor, imatinib mesylate (IM), is the current standard frontline therapy for newly diagnosed CML and it has significantly improved the prognosis of these patients. In some patients, IM therapy is however complicated by the emergence of resistance particularly in more advanced phase of CML. Several mechanisms of resistance to IM have been demonstrated, including tyrosine kinase domain (TKD) mutations, amplification and overexpression of BCR-ABL gene and clonal evolution. It has become apparent that multiple factors driven by genomic alterations and mutations would contribute to IM resistance. *Aims.* Our study aimed to identify recurrent genomic aberrations in addition to TKD mutations which could contribute to poor response to IM, primarily utilising array comparative genomic hybridization analysis (aCGH). *Methods.* DNA and RNA samples were extracted from 165 archived cells lysates collected at different time points from 37 CML patients. All patients had prior information on response to IM based on clinical, cytogenetics and BCR-ABL transcript levels. Only 103 samples were however of adequate RNA quality for TKD mutation analysis. Sixteen patients with suboptimal response to IM were subsequently identified (4 with TKD mutations and 12 without), and their 40 serial DNA samples were subjected to aCGH analysis using human genome CGH microarray 44K chips (Agilent Technologies) at a resolution of 1Mbp. Intensity data between the test and reference was processed using Feature Extraction Image Analysis Software. Confirmation by fluorescence *in situ* hybridization (FISH) using corresponding commercial probe was carried out depending on availability of metaphase cells preparations. *Results.* One of 40 samples showed poor quality of the test and was excluded from the study. Lesions were detected by aCGH in all studied patients at least on one time point. The most common genomic losses included the regions at 1p36, 1q31, 2q21, 9q34, 21q, -Y and gains at 8q24, 9q34, 14q, 16p and 22q11. Multiple genomic aberration involving gain of 8q24, losses of 9q24 and 22q11, and loss of ASS gene simultaneously were detected in one of the patient that co-harbored multiple TKD mutations (Y253F; Y253F/F359V/1023G>A). Gain of 8q24 was also noted in serial samples from 2 other patients, one of whom had a G250E TKD mutation. All patients with 8q24 gain showed progressive disease and short survival. *Conclusion.* Our findings show that multiple cryptic unbalanced genomic aberrations occur in patients with suboptimal response to IM. We were however unable to unequivocally show any single recurrent aberration that was associated with IM resistance although certain lesions, in particular, gains of 8q24 is associated with rapid disease progression of CML.

0814

NILOTINIB 400 MG BID DAILY AS FRONTLINE THERAPY OF PH + CHRONIC MYELOID LEUKEMIA: DOSE DELIVERY AND SAFETY PROFILE AT 2 YEARS

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Background. Nilotinib is an effective and registered treatment of chronic myeloid leukemia (CML) after imatinib failure. Its efficacy as frontline treatment has been explored in phase II trials from GIMEMA CML WP (Rosti *et al.*, Blood 2009: results at 12 months) and MDACC (Cortes *et al.*, J Clin Oncol 2010: median follow-up 17 months), and in a phase III trial (ENESTnd, ASH Meeting 2009: median observation 14 months). Both 300 mg and 400 mg nilotinib BID induced high and early rates of major molecular response (MMR) and complete cytogenetic response (CCgR), with a favorable tolerability profile. *Aims.* To present a detailed analysis of the dose delivery and the safety profile of nilotinib 400 mg BID in Ph-positive CML in early chronic phase (ECP) after the first year of treatment, with a minimum follow-up of 24 months. *Methods.* A multicentric phase II trial was conducted by the GIMEMA CML Working Party (ClinicalTrials.gov.NCT00481052). By March, 2010 all the patients will complete 24 months on treatment. *Results.* 73 patients received an initial nilotinib dose of 400 mg BID; the median age was 51 yrs; 21/73 patients (29%) were ≥ 65 year old at

enrollment. The median follow-up is currently 724 days. The CCgR rate at 6,12 and 18 months was 96%. Overall, the treatment was interrupted at least once in 38 patients (52%; overall, 100 interruptions), with a median cumulative duration of drug interruption of 19 days per patient (range 3-208 days); 35 pts (48%) received the full prescribed dose. The proportion of patients with ≥1 interruption decreased during the study: 45%, 22% and 7% during the periods 0-6 months, 7-12 months and 12-18 months, respectively. During the first 12 months, the mean daily dose was 600-800 mg, 400-599 mg, and less than 400 mg in 74%, 18% and 8% of patients, respectively. During the period 13-18 months the mean daily dose was 600-800 mg, 400-599 mg, and less than 400 mg in 70%, 23% and 7% of evaluable patients, respectively. At 18 months, the last daily dose was 800 mg, 400 mg and 200 mg in 68%, 25% and 7% of evaluable patients, respectively. Four adverse events accounted for the great majority of dose interruptions: bilirubin increase, skin rash and/or pruritus, amylase and/or lipase increase, transaminases increase. Only 4 events of peripheral edema have been recorded so far (3 events grade 1; 1 event grade 2); no pleural or pericardial effusion. The transient hyperglicemia did not lead to any treatment discontinuation. The hematopoietic toxicity (grade 3-4) was negligible: only 7 events (3 neutropenias and 4 thrombocytopenias) in 5 pts (7%). Two patients went off treatment after 9 and 15 months due to recurrent episodes of amylase and/or lipase increase without pancreatitis (normal ECO scan and MRI). Both patients were in CCgR, and maintained CCgR on imatinib 400 mg daily. *Conclusions.* Nilotinib 400 mg BID daily is feasible and safe in ECP CML. Acknowledgements. BolognAIL, Fondazione del Monte di Bologna e Ravenna, PRIN 2005, PRIN 2007, University of Bologna, European LeukemiaNet

0815

IN CHRONIC MYELOID LEUKEMIA PATIENTS WHO ARE IN MAJOR MOLECULAR RESPONSE AFTER 12 MONTHS OF FIRST-LINE NILOTINIB THERAPY, LOW-LEVEL BCR-ABL KINASE DOMAIN MUTATIONS ARE VERY RARE

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Background. Bcr-Abl kinase domain (KD) mutations have been documented in a variable proportion of residual Ph+ cells in some patients in complete cytogenetic response to imatinib, but their clinical significance is controversial. Nilotinib is an imatinib derivative more selective and more potent in Bcr-Abl inhibition - with reported major molecular response (MMR) rates of 81-85% at 12 months. *Aims.* We aimed to assess whether low-level Bcr-Abl KD mutations are detectable after 12 months of nilotinib treatment. *Methods.* We retrospectively analyzed the samples collected after 12 months of nilotinib treatment from 8 patients (4 males and 4 females, median age 52 years) enrolled on the GIMEMA CML working party study of nilotinib 400 mg BID as frontline treatment of CML. These patients had all achieved MMR (>3-log reduction in Bcr-Abl transcript according to the IS) between 3 and 6 months from nilotinib start and had a Bcr-Abl/Abl ratio ranging from 0.009%I^S and 0.02%^{IS} at the time of analysis. In all 8 patients, MMR was maintained at last follow up (24 months after nilotinib start), with two patients achieving complete molecular response (CMR; >4.5-log reduction) at 18 months and one at 24 months. Patients were equally distributed across Sokal risk categories (low Sokal risk, n=3; intermediate Sokal risk, n=2; high Sokal risk, n=3). Screening for low level mutations was performed by cloning the Bcr-Abl KD (a.a.240-502) in a bacterial vector and sequencing 100 independent clones for each patient. To rule out false positive results, a mutation was considered to be present in a sample if it was detected on both strands of two or more independent clones. The KD of Abl in 3 healthy individuals was analyzed in parallel. *Results.* Our cloning and sequencing approach showed evidence of Bcr-Abl KD mutations in only 1 out of 8 patients analyzed. In this high Sokal risk patient, a Q346L mutation was detected in 3/100 independent clones, and an additional T315I mutation was present in 2 out of these 3 clones. The Q346L has never been reported in imatinib-resistant patients, neither is it among the mutants emerged in the *in vitro* random mutagenesis screenings for nilotinib-resistant mutations - hence it should be devoid of any clinical relevance. The T315I, in contrast, is known to be highly insensitive to nilotinib both *in vitro* and *in vivo*. Nevertheless, Bcr-Abl transcript level continued to decline in this patient down to CMR (24 months from nilotinib start). The remaining 7 patients scored negative for mutations - showing only evidence of some single, mutated clones as also the three healthy individuals did. *Conclusions.* Our results suggest that a) low level Bcr-Abl KD mutations seem to be very rare in patients in MMR after 12 months of nilotinib therapy - a milestone achieved by the vast majority of patients; b) as hypothesized by some authors, tyrosine kinase inhibitor-resistant mutations at low levels do not always predict for subsequent relapse and should not trigger changes in therapy.

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0816

CORRELATION OF HOCT1 M420DEL SNP WITH THE CLINICAL OUTCOME OF CHRONIC MYELOID LEUKAEMIA PATIENTS TREATED WITH IMATINIB

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Background. Membrane transporters are major determinants of drug pharmacokinetics and pharmacodynamics. The human organic cation transporter 1 (hOCT1), an influx transporter, transports imatinib into chronic myeloid leukaemia (CML) cells. We and others have shown that the level of expression or functional activity of hOCT1 is a powerful predictor of clinical response to imatinib (Wang *et al.*, Clin. Pharm. Therapeut. 2008, White *et al.*, Blood 2007). hOCT1 has also been shown to exhibit several single nucleotide polymorphisms (SNPs). Genetic polymorphisms affecting the expression and transport activity may contribute to inter-individual variations in clinical responses. SNPs in hOCT1, particularly M420del (c.1260del), can affect the action and pharmacokinetics of metformin, an anti-diabetic drug and a well known hOCT1 substrate (Shu *et al.*, Clin. Pharm. Therapeut. 2008). Several alternative SNP have been described at M420, the most common of which is a 3-base deletion that results in deletion of methionine from the resultant hOCT1 protein. Aim. Our aim was to analyse hOCT1-M420del and correlate with clinical response to imatinib in CML patients. *Methods.* Genomic DNA samples were prepared from whole peripheral blood or from fresh leukapheresis samples from 174 CML patients prior to or immediately after commencing imatinib therapy at our centre. The M420del was detected by PCR-pyrosequencing. Direct DNA sequencing was used in a number of cases to reconfirm the *Results.* Twenty samples were also cloned and individual colonies were sequenced. *Results.* A total of 48 patients had a SNP detected at M420 of the 174 screened. All of these were the 3-base deletion resulting in M420del (43 heterozygous; 5 homozygous); no cases of single base deletions or changes were seen. This gives an allele frequency of M420del of 15.2%, which is similar to a previously reported frequency in Caucasians of 18.5%. Clinical information is currently available for 125 CML patients (93 carrying the normal hOCT1 gene, 29 heterozygous and 3 homozygous for M420del). For statistical analysis, cases carrying M420del were grouped together. Survival analysis showed that cases carrying exclusively the normal gene had a 5 year progression free survival of 82.8%, compared to 65.6% for cases carrying M420del (P=0.03, log rank correlation, Mantel-Cox). However, no significant difference was seen in event-free survival between the 2 groups. Summary/*Conclusions.* Our data demonstrate the clinical importance of the M420del SNP on outcome of imatinib treatment in CML. Its adverse effect on progression-free but not event-free survival is intriguing, but may be because the higher number of M420del patients who progress early are not at risk for subsequent imatinib failure within chronic phase. The data are compatible with the view that the rapid early attainment of high intracellular imatinib levels may be critical to minimise disease progression, which is less readily achieved in the presence of M420del. These data are currently being extended to a larger dataset of over 330 cases.

0817

THE ROLE OF IMATINIB PLASMA LEVEL TEST IN EVALUATION OF THE NONADHERENCE TO THERAPY IN CHRONIC MYELOGENOUS LEUKEMIA PATIENTS

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Patients (Pts) adherence to therapy is critically important for optimal outcomes in imatinib (IM) treated CML. The aim. of this report is to evaluate the significance of IM plasma level (IPL) test in the estimation of the adherence to IM treatment in CML Pts. *Methods.* Trough plasma concentrations of imatinib (IMC) were detected in 551 samples of 442 CML Pts (CP - 395, AP - 48) aged 18 or over, male/female ratio - 201/241. Blood samples were collected 24±3h after the last IM dose at 300(n=8), 400(n=337), 600(n=155)mg QD and 12±3h after the last IM dose at 800(n=51)mg BD. All Pts have signed informed consent before blood sampling. IMC were determined by a validated LC/MS/MS method. In large CML Pts population studies in I, II and III phases of clinical trials at 400mg QD, 600mg QD and 800mg BD IM doses the lowest means of IMC were 180, 350 and 1020ng/mL respectively. CML Pts with means of IMC levels less than the lowest revealed means were regarded as nonadherent in our study. *Results.* IPL test (n=406) in CML Pts with IM failure, suboptimal response or loss of response have revealed that the median of IMC in Pts at IM dose 400mg QD(n=242) was 963[0-5140]ng/mL; 8(3,3%) Pts had demonstrated IMC less than 180ng/mL. The median of IMC in Pts at IM dose 600mg QD(n=124) was 1369[2,5-3813]ng/mL; 7(5,6%)Pts have demonstrated IMC less than 350ng/mL. The median of IMC in Pts at IM dose 800mg BD(n=40) was 1845[5-5089]ng/mL; 9(22,5%)Pts have shown IMC less than 1020ng/mL. IPL tests (n= 66) were performed in CML Pts when the physician supposed the nonadherence to therapy. In this cohort the median of IMC in Pts at IM dose 400mg QD(n=46) was 861[0-3394]; 4(8,5%)Pts have demonstrated IMC less than 180ng/mL. The median of IMC in Pts at IM dose 600mg QD(n=14) was 1287[400-3813]; the Pts with IMC less than 350ng/mL were not revealed. The median of IMC in Pts at IM dose 800mg BD(n=6) was 1287[215-3813]ng/mL; 2(33%)Pts have shown IMC less than 1020 ng/mL. IPL tests for Pts with suspicion of drug-drug interaction were performed in 13 cases. The median of IMC in Pts at IM dose 400 mg QD(n=10) was 861[0-3394]; 2(20%)Pts have demonstrated IMC less than 180ng/mL. IMC in 2 Pts at IM dose 600 mg QD(n=14) were 2940 and 3635 ng/mL, in 1 patient at IM dose 800 mg BD was 1580 ng/mL and the Pts with IMC less than 350 and 1020 ng/mL respectively were not revealed. In Pts with adverse events (n=66) the median of IMC at IM dose 300mg QD(n=8) was 699[274-1550] ng/mL, at IM dose 400mg QD(n=39) - 967[269-3827] ng/mL, at IM dose 600mg QD(n=15) - 1223[544-3460] ng/mL and at IM dose 800 mg BD (n=4) - 921[381-2470]ng/mL. Only 2 Pts at IM dose 800 mg BD were nonadherent. *Conclusions.* IMC less than referent means were revealed in 32(5,8%) cases: among Pts at IM dose 400 mg QD in 14(4,2%), 600 mg QD-7(6,5%), 800 mg BD-13(25,5%) cases. IPL test is indicative of the adherence to Imatinib treatment in CML Pts.

0818

RETROSPECTIVE ANALYSIS OF PROGNOSTIC FACTORS AND OUTCOME IN AN IMATINIB TREATED CML POPULATION FROM WEST OF SCOTLAND AND LOTHIAN

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Background. Imatinib is an effective therapy for newly diagnosed patients with chronic myeloid leukaemia (CML) in chronic phase (CP) as shown in the IRIS trial. However, about 20% of patients in IRIS were censored for reasons including unsatisfactory response or adverse events, thus potentially excluding patients with a worse prognosis from outcome analysis. Two population based studies (Lucas *et al.,* Leukemia 22 1963-66, 2008; de Lavallade *et al.,* J Clin Oncol 26 3358-63, 2008) have recently shown that when these patients are included in such analyses, event-free survival (EFS) and complete cytogenetic response rate (CCyR) to imatinib are lower than those reported in IRIS. *Aims.* To assess prognostic factors and outcome in a population of CML patients treated with imatinib and compare our results to both IRIS and published population based studies. *Methods.* Data from 120 consecutive patients with CML diagnosed since 2004 in the West of Scotland and Lothian were collected retrospectively from case notes. Only patients who received front line therapy with imatinib at 400 mg/day (adjusted to tolerance and response) were considered for the study. CCyR, overall survival (OS) and EFS rates were calculated according to the Kaplan-Meier method using the log-rank test for group comparisons. A p value <0.05 was considered significant. *Results.* Median follow-up was 32.9 months (range 0.4-69.1). At the time of analysis 46.2% of the patients had discontinued imatinib after a median time of 12 months (range 0.3 - 52.7). Reasons for discontinuation included adverse events (17.6%), failure to respond (7.6%), progression (8.4%), suboptimal response (5%), death (1.7%), elective stem cell transplantation (5.9%). 5 years estimated rates of CCyR and loss of CCyR were 88.2 and 10.8%, respectively. The 5 years OS and EFS (defined as in IRIS) were 90.3 and 82.1%, respectively, consistent with IRIS data. However, when imatinib discontinuation secondary to adverse events/failure to respond are included as imatinib failures, the EFS is 53.6%. Sokal score was significantly associated with improved CCyR (at 5 years 100, 93.5 and 80.8% for low, intermediate and high risk P=0.01). Grade III/IV haematological toxicity on imatinib was associated with poorer 5 years EFS (85.8 *vs.* 60.9% for good *vs.* poor haematological tolerability, P=0.03) and CCyR rate (90.4 *vs.* 67.6% for good *vs.* poor, P= 0.01). Achieving CCyR by 12 months was associated with a non-significant trend towards improved 5 years EFS (90.2 *vs.* 69.6%, P=0.17), but not OS (95.8 *vs.* 93.3%, ns). Conclusions. Our results confirm the clinical value of imatinib, but also show that its efficacy and tolerability are less than those reported in the IRIS study and that caution must be exercised when extrapolating trial results to clinical practice. Sokal score maintained its predictive value. Achieving CCyR by 12 months was not predictive of outcome in our series, probably reflecting early introduction of second generation tyrosine kinase inhibitors in patients with suboptimal/failure to respond in our cohort. Poor haematological tolerance to imatinib appears to be a prognostic factor which might help early identification of a high risk group that warrants a more vigilant clinical approach.

0819

RESPONSE TO NILOTINIB IN PATIENTS WITH IMATINIB-RESISTANT OR -INTOLERANT CHRONIC MYELOID LEUKEMIA IN CHRONIC PHASE (CML-CP) WITH DIFFERENT BCR-ABL TRANSCRIPT TYPES

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Background. Philadelphia chromosome positive (Ph+) chronic myeloid leukemia (CML) is characterized by the BCR-ABL fusion gene which

arises from a reciprocal translocation of most of the cellular ABL gene on chromosome 9 to the BCR gene on chromosome 22. Various breakpoints in the BCR and ABL genes have been described although in most cases BCR exon 13 or 14 is fused to the ABL exon 2 (a2), resulting in the β2α2 and β3α2 transcripts, respectively. *Aims.* Variations in BCR-ABL transcript types may result in differences in disease prognosis and response to therapy. Consequently, an analysis was conducted to investigate the correlation between BCR-ABL transcript type and responses to nilotinib in the second-line setting. *Methods.* Imatinib-resistant or -intolerant patients with Ph^+ CML-CP (N = 321) enrolled on the phase 2 registration trial for nilotinib were included. BCR-ABL transcript types were analyzed in 301 (94%) patients in order to determine if transcript type influenced response dynamics. In addition, the BCR-ABL transcript dynamics were modeled as previously described (Stein *et al*. Blood. 2009;114(22):209). *Results.* Median exposure to nilotinib was 561 days. The majority of patients (95%) had typical β3α2 (63%) and β2α2 (32%) BCR-ABL transcript types; 3% of patients had both β3α2 and β2α2 transcripts and 2% had atypical transcripts (e1a2, e19a2, b3a3). Patients with β3α2 transcripts had a higher incidence of baseline mutations compared with patients with β2α2 transcripts (46% *vs.* 32%, P=.03). Transcript type did not influence response to nilotinib treatment (Table). A two-sample χ^2 test of the four endpoints (CHR, CyR, MMR, and EFS) did not show a significant difference (P>.05) between the β2α2 and β3α2 populations. Modeling results demonstrated no statistically significant difference in the response dynamics between the major BCR-ABL transcript types. *Conclusion.* In this analysis, nilotinib was shown to be effective regardless of transcript type, including in patients with rare atypical transcripts. Patients with typical β3α2 and β2α2 transcripts had similar patterns of response to nilotinib in the second-line setting.

Table.

Best Response to Nilotinib by Transcript Type	Number	Hematologic Response, %	Cytogenetic Response, %		Molecular Response, %	Event Free Survival, %	Baseline Mutation	
Transcript type	n	CHR	CCyR	PCyR	MMR	EFS	n	%
b2a2	95	87.4	44.2	13.7	16.8	58.3	85	31.8
b3a2	190	80.5	34.2	10.0	15.3	51.4	179	45.8
b2a2 + b3a2	10	90.0	50.0	20.0	10.0	71.4	8	37.5
e1a2	2	100.0	0	50.0	--	100.0	2	0
e19a2	2	50.0	0	0	--	0	2	50.0
b3a3	2	100.0	100.0	0	--	100.0	2	0
P-value: comparing b2a2 vs b3a2 using χ^2 2-sample test		0.15	0.09		0.73	0.34	0.03	

0820

ADHERENCE TO TYROSINE KINASE INHIBITORS (TKI) IN CHRONIC MYELOID LEUKEMIA (CML) SEEMS TO BE RELATED TO DURATION OF TREATMENT AND TYPE OF TKI

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Background. Chronic myeloid leukemia (CML) is a mieloproliferative disease, characterized by the Philadelphia chromosome, resulting from reciprocal translocation of chromosomes 9 and 22. Tyrosine kinases Inhibitors (TKI) of first and second generation are presently the choice of treatment for this disease and adherence to the treatment is an important factor to obtain good therapeutically *Results.* The most accepted definition of adherence is: "the extent to which a person's behavior - taking medication, following a diet, and/or executing lifestyle changes, corresponds with agreed recommendations from a health care provider". *Aims.* The objective of this study was to evaluate the adherence to TKI in patients with CML and to identify factors that can affect adherence to TKI treatment. *Methods.* A retrospective and observational analysis was performed with 131 patients taking TKI at Hematology and Hemotherapy Center, University of Campinas, Brazil. The adherence was calculated using the mean medication possession ratio (MPR), calculated as total days' dose of TKI divided by the number of the days in the observation time. This evaluation was done for about six months and correlated with other variables. To the statistical analyses were used Sperman's rank correlation and Wilcoxon rank sun test with continuity correction. *Results.* A total of 131 patients were evaluated: 92 (70.23%) were taking Imatinib, 21 (16.03%) Dasatinib and 18 (13.74%) Nilotinib. The mean age was 50 years. The majority of patients were male (61.83%) and the mean of TKI treatment time was 41 months (range: 7 to 108 months). There were 100 (76.34%) patients in first line treatment, 28 (21.73%) second line and 3 (2.29%) third line. The mean of adherence was 93.88% in all patients. 19.84% of patients were completely adherent with 100% of MPR. In this population, the MPR decreased in patients with longer duration of disease and duration of TKI treatment (P=0.01). The adherence was superior in patients participating in clinical trials (P=0.001) and in patients taking Nilotinib in comparison with patients taking Imatinib (P=0.01) or Dasatinib (P=0.027). However, most of patients that were taking Nilotinib were participating in clinical trials. There was no statistical significant difference in adherence regarding sex, age, socioeconomic status, marital status, level of education and TKI dose. *Conclusions.* The adherence in patients with CML taking TKI seems to be higher compared with other chronic disease. An important factor was how long patients have been diagnosed CML or treated with TKI. In our Cohort adherence behavior was different comparing kinds of TKI.

0821

MEASUREMENT OF TROUGH IMATINIB PLASMA LEVELS IN PATIENTS WITH CML DOES NOT SIGNIFICANTLY CORRELATE WITH TREATMENT RESPONSE BUT MAY BE SUCCESSFULLY USED IN SELECTED PATIENTS FOR DOSAGE ADJUSTMENT

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Background. Trough imatinib plasma levels (IPL) were shown to be associated with cytogenetic and molecular response in CML patients treated with imatinib in two major and a few smaller studies. However, other authors have not confirmed this observation. In a recently issued statement of the ELN, any recommendations on IPL have been implemented. *Aims.* In order to analyze the impact of IPL measurement on treatment results we have introduced the determination of IPL into our laboratory follow-up of CML patients in 2007. All patients have signed informed consent before blood sampling. *Methods.* Samples were obtained at routine clinical check-ups. IPL were measured by ultra-performance liquid chromatography-tandem mass spectrometry assay using UHPLC UltiMate 3000 RS (Dionex, USA) coupled with the API 4000 tandem mass spectrometer (Applied Biosystems, USA). The actual sample was eligible for analysis providing patient had been treated with imatinib for at least 18 months allowing evaluation of cytogenetic and molecular response, there was no other evident cause of the suboptimal response or failure and the sample was taken at least one month after initiation of treatment and between 24±6 hours after the ingestion of the drug. Other medication, the actual duration of the therapy, time interval between the dose and the meal ingestion, weight, body-mass index, body surface area or co-morbidity of the patients were not taken into the account. Patients pretreated with interferon were analyzed separately. Statistics were done using Spearmans correlation analysis and Mann-Whitney U-test. *Results.* 553 samples from 112 patients with CML (45 women, 55 of them pretreated with interferon) were available for analysis. Median age of patients was 56 years (range: 22-81) follow-up was 72 months (range: 18-236). 81 % of patients achieved complete cytogenetic and 58 % major molecular response. Despite significant correlation between imatinib dose and IPL CC=0.336; P<0.001 there was no significant correlation between IPL and probability of achievement of optimal cytogenetic or molecular response (at 12 and 18 months, respectively). We found high IPL inter-individual variability (log-norm distribution of data) in the range 394-3133 mg/l (min-max, outliers excluded, n=86, 24±1 hours after 400 mg dose) and intra-individual variability (CV) between 14.2 and 43.3 % (n=82, 24±2 hours after 400 mg dose). In two patients with low IPL (<900 ng/mL) dose escalation led to IPL increase (>2000 and >1400 ng/mL) and substantial improvement of treatment response. *Conclusions.* We were not able to demonstrate a clear correlation between IPL and response to imatinib therapy in our group of patients with CML. We believe that this is caused by differences in patients' compliance, leukemia biology and other variables that are difficult to eliminate in the routine clinical praxis. On the other hand in rare selected patients with low IPL and suboptimal response we were able to demonstrate that escalation of imatinib

dose lead to optimal treatment response. *Acknowledgement.* The work was supported by grants NS9627-3, NS9949-3 (Ministry of Health, the Czech Republic), MSM 6198959223 and MSM 6198959205 (Ministry of Education, Youth and Sports, the Czech Republic).

0822

ADDITIONAL CHROMOSOME ABERRATIONS IN CHRONIC MYELOID LEUKEMIA PATIENTS UNDERGOING TYROSINE KINASE INHIBITORS THERAPY

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Background. Variety of additional chromosome aberrations (ACA) is detected in chronic myeloid leukemia (CML) patients, but their influence on achieving cytogenetic response and disease prognosis is not clear. *Aims.* The aim of the present study was to detect frequency of different ACA in CML patients undergoing treatment with tyrosine kinase inhibitors and to estimate their influence on achieving cytogenetic response. *Methods.* We present a retrospective study based on 1060 patients treated for CML with inhibitors of tyrosine kinase (ITK) at National Research Center for Hematology. We performed conventional cytogenetic analysis and interphase fluorescence *in situ* hybridization (FISH) with probes LSI BCR/ABL, CEP 8, LSI 7q31/CEP 7. *Results.* ACA in Philadelphia chromosome-positive (Ph+) cells were detected in 207 patients (see the Table). At diagnosis we detected 4 cases of variant translocations and 37 cases of complex translocations involving three or four chromosomes. BCR/ABL gene in 94-100% of cells was detected in all cases. Ph-chromosome duplication was the most frequent ACA in Ph^+ cells. ACA didn't influenced significantly the disease prognosis but 35% of patients with ACA achieved major cytogenetic response (MCR) and complete cytogenetic response (CCR) later than patients without ACA: at about 12 and 24 months from the beginning of ITK treatment. In some complex karyotypes with multiple chromosome abnormalities Ph-chromosome was masked or detected in small amount of cells, but BCR/ABL gene was detected in the majority of cells. For example in case 1 karyotype 46XX, del(X)(q27), t(14;22)(p11;q11), der(9), del(8)(q27), 16p+ [20] / 46XX, t(9;22)(q34;q11) [5] showed 20% of Ph+ cells but FISH detected BCR/ABL gene in 85% of cells; in case 2 karyotype 53-56 XXY,+8,+8,+12,+15,+18,+19,+20,+21,+21, der(21), t(21;22)[25] showed absence of Ph+ cells but FISH detected BCR/ABL in 100% of cells. All cases of complex karyotypes were associated with blast crisis phase. We report two cases of dicentrics from Ph-chromosome. Patient with karyotype 47XY t[9,dic(22), +dic(22)(22pter-q11;q11-22pter)][25] showed 100% BCR/ABL-positive cells; he achieved CCR 6 months after starting nilotinib as a second line therapy. In case with karyotype 47XY, t[9,dic(22), +dic(22)(22pter-q11;q11-22pter)] [16] / 46XY, t(9;22)(q34;q11) [14] / 46XY [4] FISH detected 85% of BCR/ABL-positive cells, 40% of cells demonstrated BCR/ABL gene amplification. Escalating dose of imatinib to 800 mg reduced the amount of BCR/ABL-positive cells to 30%. ACA in Ph- cells were detected in 110 patients (see the table) in different periods after achieving MCR and CCR: Size of Ph™clones with ACA ranged from 0% to 100% and wasn't associated with treatment response and disease progression.

Table. Types of ACA detected in Ph+ and Ph-cells.

ACA in Ph-positive cells	number of cases	frequency, %
sum total	207	19,53
variant translocations	4	0,37
complex transloc. involving 3-4 chromosomes	37	3,49
+ Ph-chromosome	42	3,96
trisomy 8	32	3,02
inv (9)	21	1,98
-7 / del (7q)	11	1,04
-17 / i(17)(q10)	10	0,94
X or Y abnormalities	10	0,94
inv (3) / dup(3p)	5	0,47
- 21 / +21 / del (21q)	6	0,57
aneuploidy of other chromosomes	11	1,04
complex karyotypes	18	1,70

ACA in Ph-negative cells	number of cases	frequency, %
sum total	110	10,38
trisomy 8	32	3,02
-7 / del (7q)	10	0,94
-17 / i(17)(q10)	9	0,85
X or Y monosomy / deletion	9	0,85
inv(3)/dup(3p)	5	0,47
- 21 / del(21q)	4	0,38
aneuploidy of other chromosomes, rare translocations like t(14:22)(p11:q11), t(15:21)(q21:q21) and other abnormalities	41	3,87

Conclusions. 1. Ph+cells with ACA were detected in 20,28% of patients, Ph-ells with ACA in 10,38%. 2. 35% of patients with ACA in Ph+clones achieved MCR and CCR later then patients without ACA. 3. ACA in Ph-clones didn't influence clinical outcome. 4. Two patients with dicentrics from Ph-chromosome showed MCR and CCR on ITK therapy.

0823

BCR-ABL MUTATION ANALYSIS USING BOTH ASO-PCR AND DIRECT SEQUENCING IN NEW CHRONIC PHASE CHRONIC MYELOID LEUKEMIA PATIENTS WITH SUBOPTIMAL RESPONSE OR TREATMENT FAILURE FROM IMATINIB TREATMENT

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Background. Resistance to imatinib can occur in chronic myeloid leukemia (CML). Mutations in BCR-ABL kinase domain have been known as the clinically most relevant mechanisms of imatinib resistance. Several studies have shown that 40~80% of the imatinb resistant patients have BCR-ABL kinase domain mutations. However, there has not been much information about mutation status in CML patients with suboptimal response to imatinib. From Tyrosine Kinase Inhibitor Optimization and Selectivity (TOPS) study which investigates efficacy of imatinib 400 mg daily and 800 mg daily with Philadelphia positive CML CP patients, relationship between various responses and emergence of mutation was investigated by both standard and highly sensitive mutation assays. *Aims.* As a clinical correlative study (CCS) program with samples from TOPS study, we analyzed BCR-ABL mutation from patients with suboptimal response or treatment failure to imatinib in order to investigate their mutation status. *Methods.* Suboptimal responders and treatment failures were selected based on European Leukemia Net (ELN) 2009 guideline. CML patients' samples from the TOPS study were collected at particular time points after initiation of imatinib treatment and stored as cryopreserved cells or isolated RNAs in four regional referral laboratories. Mutations in BCR-ABL kinase domain were analyzed using direct sequencing and allele-specific oligonucleotide (ASO)-PCR.

Table. Mutation status of patients.

Response	Base line	6 months		12 months		18 months	
		mutation	Pt	mutation	Pt	mutation	Pt
Optimal		1 (5%)	21	0 (0%)	8	0 (0%)	3
Suboptimal		1 (14%)	7	1 (13%)	8	2 (50%)	4
Failed		1 (20%)	5	6 (38%)	16	4(100%)	4
Total Pt (n=104)	31	33		32		8	

Results. We analyzed total 104 samples collected from 51 patients at different time points including diagnosis, 6 months, 12 months and 18 months after initiation of imatinib treatment. All patients were in CP. We found ten BCR-ABL kinase domain point mutations including G250E, Y253H, T315I, and D444Y in 4 patients. In addition, we also found other mutations including 35 base pair insertion between exon 8 and exon 9 of ABL, and deletion of exon 7 of ABL in other 8 patients. No mutation was found from the patients' samples collected at diagnosis. At 6 months, mutation was found 5% (1 of 21), 14% (1 of 7) and 20% (1 of 5) patients in optimal response, suboptimal response and treatment failure group, respectively. ASO-PCR revealed that one patient in optimal response group had T315I. The same mutation status of the patient maintained at 12 months and the patients showed treatment failure at 12 months. At 12 months, mutation portion was 0% (0 of 8), 13% (1 of 8) and 38% (6 of 16) in optimal response, suboptimal response and treatment failure group, respectively. At 18 months, 50% (2 of 4) of suboptimal molecular responder and 100% (4 of 4) of no CCgR group showed mutations. Conclusions. Patients with suboptimal response or treatment failure showed much higher chance

of BCR-ABL point mutation, 35 base pair insertion or exon 7 deletion in comparison with optimal responders, suggesting that mutation screening is important for patients with suboptimal response as well as treatment failure on the basis of ELN guideline. Highly sensitive ASO-PCR provided early detection of point mutation in BCR-ABL kinase domain. However, clinical relevance of low level mutant clone, 35 base pair insertion and exon 7 deletion require long-term follow up for better understanding.

0824

CHARLSON COMORBIDITIES INDEX (CCI) MAY PREDICT COMPLIANCE AND DEVELOPMENT OF PLEURAL EFFUSIONS IN ELDERLY CHRONIC MYELOID LEUKEMIA (CML) RESISTANT/INTOLERANT PATIENTS TREATED WITH DASATINIB

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Background. The Charlson comorbidities index (CCI) is a list of 19 conditions (including cardiologic, pulmonary diseases, diabetes, etc) with a weight assigned from 1 to 6, derived from relative risk estimates of a proportional hazard regression model using clinical data. *Aims.* We retrospectively evaluated the weight of CCI in a cohort of 125 elderly (> 60 years) chronic phase chronic myeloid leukemia (CP-CML) patients receiving dasatinib after imatinib resistance or intolerance. *Methods.* Score point 0 was assigned to 65 patients, whereas a score point >1 was assigned to 60 patients (35 patients =1, 19 patients= 2; 5 patients= 3; 1 patient= 4). Fifty-two patients received 70 mg twice daily and 56 patients received 100 mg once daily, in accordance with the results of the phase III trial, whereas 17 patients started with a dose less than 100 mg. *Results.* We found a significant association between CCI and drug reduction or suspension rate: during dasatinib treatment 49% of score 0 patients experienced a reduction of the dose compared to 63% of patients with score 1, 74% of patients with score 2 and 100% of patients with score 3 and 5 (P=0.03). Of the 65 patients with score 0, 29% had at least one suspension (79% for haematologic and 21% for non-hematologic toxicity, respectively), compared to 46% of patients with score 1 (37% for hematologic and 69% for non-hematologic toxicity), 58% of patients with score 2 (36% for hematologic and 64% for non-haematologic toxicity) and 100% of patients with score 3 and 4 (all patients for both types of toxicity) (P=0.003). Forty-one patients (32.8%) experienced pleural effusion during treatment: of these, 17 patients (26%) had score = 0, 13 patients (40%) score=1, 8 (42%) score=2 and 3 (60%) had score 3-4 (P=0.002). More pleural effusions occurred in patients who had score > 0 and who received higher dasatinib dose (140 mg). Nine patients experienced grade 3 and 31 patients grade 1-2 effusions: although the incidence of this event correlated with CCI stratification and dose received, its severity did not show the same correlation. *Summary.* In conclusion, in elderly CML patients treated with dasatinib, the rate of drug reduction or suspension and the incidence of pleural effusions seem to be associated with the presence of comorbidities: the stratification according to CCI pre-dasatinib therapy may allow to identify patients at risk to have major toxicities.

Chronic myeloid leukemia - Clinical 3

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LOW DOSE CONTINUOUS THERAPY WITH DASATINIB SIGNIFICANTLY IMPACT ON SURVIVAL OF PATIENTS WITH CHRONIC MYELOID LEUKAEMIA (CML) RESISTANT OR INTOLERANT TO IMATINIB. RESULTS FROM A REAL LIFE-BASED ITALIAN MULTICENTER RETROSPECTIVE STUDY ON 114 UNSELECTED PATIENTS.

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Background. Dasatinib is effective in treating chronic myeloid leukemia (CML) patients resistant/intolerant to imatinib. To date, most of the data come from clinical trials on selected patients. Thus, studies based on real life are opportune. *Aims.* The aim of the study was to evaluate the safety and efficacy of Dasatinib as second line therapy for patients with CML either resistant or refractory to imatinib outside from clinical trials or compassionate use. Patients and *Methods.* 114 unselected CML patients resistant/intolerant to imatinib received dasatinib as second line therapy outside from clinical trials. All patients had chronic phase (CP-CML). Sokal score was low for 29, intermediate for 44 and high for 41 patients, and they were either resistant (n=88) or intolerant (n=16) or resistant and intolerant (n=10) to imatinib therapy. All patients were evaluated for hematologic, cytogenetic and molecular response, discontinuation/dose reduction of Dasatinib, event-free survival (EFS) and overall survival (OS). Moreover, we analyzed toxicities according to NCI-CTC, focusing on grade III-IV toxicities. *Results.* At 12 months, cumulative incidences of complete hematologic response, complete cytogenetic response (CCyR) and major molecular response were 94%, 55%, and 35%. Cumulative event-free survival (EFS) and overall survival (OS) were 91 and 93%. Noteworthy, we did not observe any survival difference between patients with low, intermediate or high Sokal score (P=0.4). We did not found any association between the dose of prior imatinib (400, 600 or 800) and the probability of achieving CCyR at 1 year with dasatinib (P=0.7). Furthermore, we did not found any association neither between the duration of the previous treatment with imatinib and the probability of achieving CCyR at 1 year with dasatinib (P=0.9). Nevertheless, we did not observe any difference between patients who received imatinib for more than 3 years with respect to those receiving it for less than 3 years (P=0.9). Finally, we did not observe any association between best response to imatinib and probability of achieving CCyR after 1 year with dasatinib (P=0.8). As a matter of fact, we observed a statistically significant better outcome in term of both DFS (P=0.04) and OS (P=0.04) for the 54 patients who received dasatinib at lower doses continuously without interruption with respect to the 60 patients receiving higher doses but forced to discontinue the treatment due to grade III-IV toxicity. *Conclusions.* We confirm the safety and efficacy of dasatinib as second line therapy for patients with CML either resistant or refractory to imatinib even outside from clinical trials. This experience challenges the wisdom that dasatinib, a drug characterized by short half life, must achieve nearly continuous target inhibition via high dose therapy for maximal clinical impact. Further studies are warranted, in order to extend the use of low dose continuous dasatinib to real life patients potentially not eligible to receive high dose dasatinib.

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IMATINIB MESYLATE INDUCES HIGH COMPLETE CYTOGENETIC AND MAJOR MOLECULAR RESPONSE RATES IN CHILDREN AND ADOLESCENTS WITH PHILADELPHIA CHROMOSOME-POSITIVE CHRONIC MYELOGENOUS LEUKAEMIA IN CHRONIC PHASE

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Background. Imatinib mesylate (IM) has been established as first line treatment for children with Philadelphia-chromosome positive (Ph^+) chronic myeloid leukemia (CML). However, results from controlled trials in children are sporadic. Aim. This study was designed to evaluate the IM efficacy in Ph^+ CML patients in chronic phase (CP) aged <18 years at diagnosis, previously untreated or resistant to Interferon (IFN). *Methods.* According to prospective guidelines, pediatric patients with a diagnosis of Ph^+ CML in CP were treated with IM at a dosage of 340 mg/sqm/day. The informed consent was obtained in all cases. Data were reported to the study center on standardized forms by the treating physician. Specimen from peripheral blood and bone marrow were assessed by cytogenetics and by quantitative PCR for BCR-ABL transcript rates in central laboratories for standardized monitoring at three months intervals. *Results.* From March 2001 to May 2009, 29 Ph^+ CML patients in CP (12 female, 17 male; median age: 1110/12 years [range:36/12-1710/12 yrs]) were recorded from 8 Italian pediatric centers. Twenty-five patients had previously received hydroxyurea and 5 of them alpha-Interferon. IM was started in all patients, including 7 with an HLA identical sibling. The median dose of IM administered was 318 mg/m^2/day. Grade >2 toxicities were observed mostly beyond the first 6 months. Seven patients experienced grade 2-3 neutropenia (n=6) and/or thrombocytopenia (n=2). Eleven patients presented grade 3-4 isolated or combined side effects: vomiting (n=1), muscle pain (n=4), diarrhea (n=1), hepatitis (n=1), pancreatitis (n=1), bone metabolism alterations (n=4), impaired longitudinal growth (n=3). Three patients (10%) stopped IM because of severe toxicities (1 crossed to dasatinib, 1 underwent stem cell transplantation -SCT- and 1 in complete molecular response didn't received any treatment). Twenty-four of 25 (96%) evaluable patients achieved complete cytogenetic response (CCyR) after a median time of 6 months (range 3-12 months), one of them had a cytogenetic relapse after 33 months. One patient was lost to follow-up in CCyR. At month 12 after starting IM, all evaluable patients were in CCyR and 10/11 (91%)presented major molecular response (MMR, defined as <0.1% BCR-ABL). To reduce disturbances of bone metabolism and longitudinal growth impairment, 8 MMR patients are receiving IM at a same daily dosage for 3 weeks a month. Overall, 8 patients underwent SCT (6 from identical siblings, 2 of them in CCyR, and 2 from matched unrelated donors, 1 in partial CyR and 1 after a cytogenetic relapse) after a median time of 6 months (range 3-51 months). All patients are alive in MMR (8 after SCT, 1 in dasatinib, 1 without any treatment and 18 still receiving IM), after a median follow-up of 40 months. *Conclusions.* In our experience, IM induces high CCyR and MMR rates in CML children and adolescents in CP, like in adults. Side effects seem tolerable, as only 10% of the total cohort stopped imatinib. Moreover, IM treatment for 3 week a month seems to improve bone metabolism and longitudinal growth in the not yet outgrown patients, without impairment on the disease outcome.

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COMPLETE MOLECULAR RESPONSE IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA ON IMATINIB THERAPY

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Background. Imatinib treatment in chronic myeloid leukemia (CML) patients leads to significant suppression of Ph^+-positive leukemia cells. Identification of patients with continuous and stable complete molecular response (CMR) defined by RQ-PCR opens a discussion of possible complete leukemia eradication. Aim. To analyze frequency and stability of CMR in early chronic phase (ECP) and late chronic phase (LCP) CML patients on imatinib therapy. *Methods.* We analyzed 863 peripheral blood samples in 2 groups of 184 chronic phase (CP) CML patients on imatinib therapy (dose 400-600 mg). 1st group: 78 ECP CML patients, median age 40 years (15-65), median time from diagnosis 50.8 months (5-99), imatinib started in median 2 (1-6) months after diagnosis. 10 of these 78 patients received imatinib combined with interferon alpha. 2nd group: 106 LCP CML patients with previous interferon alpha failure or intolerance, median age 41 years (9-68), median time from diagnosis 106.8 months (43-244). Imatinib started in median 35.5 months (12-143) after diagnosis. A standardized RQ-PCR with sensitivity 0.01% was performed 1-4 times a year (median 1.7). BCR-ABL expression results presented in international scale (IS) with samples quality approved by >1000 copies per reaction of control gene ABL. CMR dynamics was examined from 6th to 60th month therapy within 6 months intervals, patients number for every term was 20-45% from total number. *Results.* Frequency and duration of CMR in ECP and LCP CML patients were different (Table 1). In ECP group 21 (26.9%) of patients achieved CMR for the first time during 18 months of therapy: 10 (12.8%) and 11(14.1%) for 12th and 18th month of treatment consequently. CMR was achieved for 9 (90%) patients in the subgroup of 10 ECP patients receiving combination of imatinib and interferon alpha; 4 (40%) of them achieved CMR on 18th therapy month. Maximum percentage of CMR patients was observed on 30th therapy month - 21(79.2%) of 24 analyzed on that term, on the 60th therapy month later CMR rates lowered: 11 (33.3%) of 27 analyzed on that term. In LCP group most of the patients achieved CMR for the first time on 12th and 42nd month of treatment: 4(23.5%) of 17 and 7(22.6%) of 31 analyzed on that term consequently. Maximum percentage of CMR patients was observed on 18th month of treatment: 6 (54.6 %) of 11 analyzed on that term. *Conclusion*. The early start of imatinib therapy changes CMR achievement dynamics and enables the growth of CMR patients proportion and CMR duration in ECP CML patients. The decreasing number of CMR ECP patients after 30th therapy month possibly reflects the patients with the secondary resistance. Imatinib and interferon alpha combined therapy can increase the CMR patients proportion.

Table 1. Frequency and duration of CMR.

	ECP CML (n=78)		LCP CML (n=106)	
	n	%	n	%
Without CMR	39	50	65	57.50
With CMR	39	50	45	42.45
Continuous (stable) CMR	15	19.23	7	6.60
Single-time CMR, no follow-up	9	11.54	9	8.49
CMR lost up to MMR once and restored to CMR again	7	8.97	16	15.09
CMR lost to the levels exceeding MMR	8	10.26	13	12.26
Median of CML duration, months	22		14	

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C-MYC SCORE IN CHRONIC MYELOID LEUKAEMIA: A NEW BIOLOGICAL MARKER OF DISEASE PROGRESSION

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Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disorder characterized by the Philadelphia chromosome. The disease runs a chronic phase followed by an accelerated phase and ends by an acute blastic crisis. The mechanisms underlying disease progression include molecular events that involve the action of signaling pathways by BCR-ABL, resistance to apoptosis in both accelerated and blastic phases, differentiation blockade, decreased immune surveillance as well as drug resistance. C-myc is considered a key gene in many cancers, yet the clinical relevance of c-myc score in CML has not yet been investigated. The aim of the present study was to assess c-myc score and its value in predicting disease progression. Forty consecutive patients with CML were enrolled. Diagnosis was established by complete blood picture, bone marrow, leukocyte alkaline phosphatase score, RT-PCR for detection of BCR-ABL fusion gene. Risk stratification of patients was calculated using both scoring systems according to Sokal and Euro score. C-myc detection was carried out immunohistochemically on peripheral blood smears and scored according to Kyriakou. Patients in accelerated and blastic phase had a statistically significant higher mean c-myc score compared to those in chronic phase (t=6.09, P=0.000). Patients with Sokal stage 3 and high risk by Euro score had the highest c-myc score (F=19.57 and F=16.14, P=0.000 respectively). Those who transformed throughout the study period had significantly higher c-myc value (t=2.28,P=0.02). We established a cut-off value of c-myc score, accordingly patients scoring 300-350 had a better overall survival compared to those who scored 350-400. An inverse relationship was found between c-myc score and overall survival (r= -0.812,P=0.000) . Moreover, patients having a lymphatic blast crisis exhibited a significantly higher c-myc score compared to those in myeloid crisis (t=2.11,P=0.03). In conclusion, c-myc scoring could be a reliable marker of disease progression in CML. Its cut-off value is a good prognostic indicator of the blastic lineage and of overall survival. A new clinical scoring system incorporating c-myc score is envisaged.

0830

IMPACT OF BCR-ABL MUTATIONS ON RESPONSE TO DASATINIB IN ELDERLY CHRONIC MYELOID LEUKEMIA PATIENTS RESISTANT TO IMATINIB

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Background. Mutations of the BCR-ABL kinase domain represent a frequent cause of Imatinib (IM) resistance in chronic myeloid leukemia (CML) patients (pts). Dasatinib (DS) is highly effective against the majority of BCR-ABL mutants detected in pts failing IM. However, little is known about the incidence of mutations in elderly CML pts resistant to IM and on their impact on response to DS in this setting. Aim and Methods. To evaluate the role of pre-DS BCR-ABL mutations, we analysed 81 pts aged >60 years who received DS for chronic phase (CP) CML resistant to IM. Forty-five pts (55.5%) did not display any BCR-ABL mutation, while 36 (44.5%) had one or more mutations. Twenty-four different mutations were detected in the 36 mutated pts, with 4 cases displaying multiple mutated clones. Most frequent mutations were: M244V (n=7), Y253H (n=4), F317L (n=3), F359V (n=5), M531I (n=3) and H396R (n=3). Overall, these 6 mutations represented 69% of all cases. V299L and T315I mutations insensitive to DS were detected in one case each. Results. The mutated and non-mutated groups were comparable for sex distribution, age at DS start, concomitant diseases, medications and cause of IM resistance. DS daily starting dose was higher in mutated pts (140 mg in 24 pts, 100 mg in 11 pts, < 100 mg in 1 pt) than in non-mutated ones (140 mg in 13 pts, 100 mg in 27 pts, < 100 mg in 5 pts). Consequently, the dose reduction rate, owing to toxicity, was higher in the mutated pts (26/36, 72% *vs.* 19/45, 42%; P=0.01). Final DS dose was almost identical in the two groups (mean dose: 90 mg in mutated and 100 mg in non-mutated), as was the percentage of pts that suspended therapy and the rate of adverse events (including pleural effusion). When considering best response to DS therapy, non-mutated patients did significantly better than mutated ones. Complete cytogenetic responses (CCyR) and/or major/complete molecular responses (MMolR and CMolR) were obtained in 24 of the 39 evaluable non-mutated patients (61.5%) and in 11 of the 35 evaluable mutated patients (31.5%) (P=0.02). The rate of primary resistance was higher in the mutated group (16/36 *vs.* 7/45, P=0.009), and even excluding the 5 pts with mutations unresponsive to DS (i.e. 3 with F317V, 1 with V299L and 1 with T315I), mutated pts had a lower CCyR/MolR rate (11/31 *vs.* 24/39, P=0.05) and more frequently displayed primary resistance (13/31 *vs.* 7/45, P=0.02). On the contrary, secondary resistance (4/36 *vs.* 0/45), severe toxicities (4/36 *vs.* 6/45) and suspensions due to other causes (2/36 *vs.* 5/45) did not significantly differ in the two cohorts. Summary. Our data suggest that BCR-ABL mutations account for roughly half of IM resistance in elderly CP-CML pts, a proportion similar to that found in the general population. In the elderly setting, however, presence of pre-DS BCR-ABL mutations seems to reduce optimal response to therapy, in terms of lower CCyR and MolR rates.

0831

DYNAMICS OF CHRONIC MYELOID LEUKEMIA UNDER NILOTINIB THERAPY

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Background. Understanding the molecular mechanisms behind the appearance and clonal expansion of BCR-ABL expressing cells in chronic myeloid leukemia (CML) has transformed therapy and prognosis for this disease. Rationally designed tyrosine kinase inhibitors such as imatinib and nilotinib lead to deep and durable responses and have reduced the risk of progression to blast crisis. Although the current standard of care is imatinib, nilotinib may give superior results with the advantage of efficacy in the presence of mutations that render imatinib inactive. *Aim.* In this work, we utilized serial quantitative estimation of BCR-ABL transcripts from patients with newly diagnosed CML and treated with nilotinib to understand the dynamics of the response and why responses with this agent are faster compared to imatinib. *Methods.* The GIMEMA group has conducted a Phase II trial of nilotinib in a cohort of 73 patients with early chronic phase CML (Rosti *et al.* Blood 114:4933, 2009). Serial quantitative RT-PCR data of BCR-ABL were recorded and fitted to a mathematical model of hematopoiesis that has been used to understand CML dynamics under imatinib therapy (Dingli *et al.*, Clin Leukemia 2:133, 2008, Lenaerts *et al.*, Haematologica, in press 2010). The model was fitted to the data using a non-linear least squares approach with constraints based on established observations. The fraction of cells responding to nilotinib and the effect of the drug on the behavior of CML progenitors was determined. *Results.* The fraction of CML cells responding to nilotinib is 2.4% which is lower than estimates for imatinib (4.6-5.0%). BCR-ABL expression enhances the self-renewal of progenitor cells. Comparison between Model and data suggests that nilotinib effectively reduces self-renewal propensity of treated compared to normal progenitor cells (epsilon_CML=0.72, epsilon_normal=0.84, epsilon_IMAT=0.9, epsilon_NIL=0.92). This results in a significant fitness disadvantage on the CML progenitor cells (f_CML=2.2, f_normal=1, f_IMAT=0.63, f_NIL=0.49), leading to their washout from hematopoiesis and a return to blood formation dominated by normal cells. *Conclusion.* The reduction in reproductive fitness of CML progenitor cells achieved with nilotinib is higher than what is

achieved by imatinib. This explains the faster and deeper response observed in patients with CML treated with nilotinib. By reducing the fitness advantage of CML cells, nilotinib enables Darwinian selection within hematopoiesis to effectively eliminate the leukemic population.

0832

PROJECTING THE LONG-TERM SURVIVAL OF NEWLY DIAGNOSED PATIENTS WITH CHRONIC MYELOID LEUKEMIA (CML) IN CHRONIC PHASE (CP) RECEIVING NILOTINIB OR IMATINIB

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Background. The international randomized Phase III ENESTnd Trial has recently demonstrated that CML-CP patients who received nilotinib 300mg BID or 400mg BID experienced significantly higher rates of major molecular response (MMR) and complete cytogenetic response (CCyR) after 12 months than patients receiving imatinib 400 mg QD. In this head to head trial, significantly fewer patients on nilotinib progressed to accelerated phase or blast crisis (AP/BC) compared with imatinib (0.7% nilotinib 300 mg BID v. 3.9% imatinib [log-rank test of time-to AP/BC p-value is 0.0095], and 0.4% nilotinib 400 mg BID v. 3.9% imatinib [log-rank test of time-to AP/BC P=0.0037]). Survival prognosis in AP/BC is significantly worse compared with CP, thus preventing or delaying progression is expected to translate into survival gains. *Aims.* The present study modeled the survival of newly diagnosed CML-CP patients receiving first line therapy with nilotinib 300mg BID or imatinib 400 mg to infer long term outcomes. *Methods.* A three-state Markov model linking the rate of conversion from CP to AP/BC to mortality was developed to project the survival for a representative CML-CP patient enrolled in the ENESTnd trial (male, average age 47 years at treatment initiation). To project overall survival and conversion rates to AP/BC over time, a commonly used survival model (Weibull) was fitted to the 12-month Kaplan-Meier curve of transformation from CP to AP/BC from the ENESTnd trial. The model assumed that patients who have not progressed are not at risk for CML-related death but experience the age-specific mortality of the general population. Patients progressing to AP/BC were assumed to switch therapy to dasatinib 140 mg/day. These patients' survival was then simulated using data from published trial literature. *Results.* The Weibull model fit the data well: the sum of squared difference between the observed and predicted rates of progression at 3, 6, 9 and 12 months was 0.000958. The model-projected life expectancy for a representative newly diagnosed CP-CML patient was 22.76 years for imatinib. In comparison, the corresponding estimate was 31.59 years for nilotinib. This resulted in a projected survival improvement of 8.83 additional years for nilotinib compared to imatinib. To put these estimates in perspective, a 47-year old British man without CML would be expected to live approximately 32.56 years (source: 2008 UK Interim Life Tables). *Conclusion.* This long-term survival model projection based on the clinical trial data resulted in an estimated improvement in life expectancies for patients initiated on nilotinib 600 mg/day compared with imatinib 400mg/day as first line therapy for CML-CP. These projections require confirmation and validation through actual longer-term observations that are ongoing. The analysis suggests that the reduction in rates of progression to AP/BC observed in the ENESTnd trial with nilotinib as first line therapy will translate into survival gains over first-line therapy with imatinib. This analysis also suggests that the use of nilotinib may improve life expectancy of CML-CP patients close to the life expectancy of the general, age-matched population.

0833

SECOND-GENERATION TYROSINE KINASE INHIBITORS TREATMENT CHANGES THE PROGNOSIS IN CML PATIENTS WITH ADDITIONAL PH-CHROMOSOME

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Introduction. Clonal evolution is defined as additional chromosomal abnormalities (ACA) in Ph-positive cells. Such karyotypic abnormalities were described more than 30 years ago and can be found in more than 70% patients in advanced disease stages. The most frequent ACA, the second Ph-chromosome, is associated with disease progression. The ACA in Ph-positive cells during imatinib treatment is associated with more rapid CML progression, while it's appearance in Ph-negative cells has no adverse prognostic impact, since 50”'75% such patients achieve major or complete cytogenetic response. The study goal. Estimate the possibility of clonal evolution supression at imatinib treatment with the help of second-generation tyrosine kinase inhibitors (TKI). Materials and *methods.* The complete blood counts, morphological and cytogenetic studies of bone marrow cells were done in 393 CML patients. The median follow-up is 94 months. *Results and discussion.* The ACA in Ph-positive cells were revealed by conventional cytogenetic study in 50 (13%) of 393 CML patients. The most frequent of them was the additional Ph-chromosome, found in 60% cases (30 of 50 patients). The majority of these patients was in chronic phase (16 patients), 6 “' in accelerated phase and 8 “' in blastic phase. Seventeen patients had additional Ph-chromosome alone, while 13 “' in association with other abnormalities: trisomy 8 (8 cases), del 7 or monosomy 7 (3 cases) and complex abnormalities (2 cases - in accelerated and blastic phase). There was evident male predominance (M:F=23:7). At the moment when additional Ph-chromosome was found the median age of our patients was 50 years (16”'61), the previous treatment duration (hydroxyurea, busulfan, 6-mercaptopurine, combined chemotherapy, interferon) was rather long (median 5 years). At present 43% (13 of 30) patients have died: 11% (2) in chronic, 50% (3) in accelerated and all 8 patients in blastic phase. The CML chronic phase patients are the most interesting from prognostical standpoint. The duration of imatinib treatment in 16 patients with additional Ph-chromosome varied from 6 months to 5,5 years. The follow-up duration of patients with additional Ph-chromosome varies from 26 to 176 months (median 107 months). All 16 patients have achieved complete hematological response after 3 to 6 months of imatinib treatment, but all patients receiving standard imatinib doses had primary cytogenetic resistance. Only 13% patients (2 of 16) have achieved cytogenetic response after escalation of imatinib dose to 600”'800 mg daily (CCyR - 1, PCyR - 1). Due to imatinib resistance 14 patients were switched to second-generation TKI (dasatinib - 10, nilotinib - 3, bosutinib - 1 patient). Three of these patients later progressed to accelerated phase; in 2 of them we have found T315I mutation. All of the 11 patients still in chronic phase have achieved complete hematological response; PCyR was found in 45%, and CCyR in 36% cases. Conclusion. The additional Ph-chromosome is associated with cytogenetic resistance to both standard (400 md daily) and high (600”'800 mg daily) doses of imatinib. The dose escalation was ineffective in the majority of cases. The second-generation of TKI allows to restore Ph-negative hematopoiesis, suppress the subclone with additional Ph-chromosome and achieve PCyR in 45% and CCyR in 36% patients. This approach should be tested in large-scale studies. The male predominance in patients with doubling of Ph-chromosome also needs further investigation.

0834

MEGAKARYOCYTIC BLASTIC PHASE (BP) OF CHRONIC MYELOID LEUKEMIA (CML) - A SINGLE INSTITUTION EXPERIENCE

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Background. BP transformation of CML with megakaryocytic phenotype is uncommon, accounting for <3% of all CML BP (Faderi S, Ann Intern Med. 1999;131:207-19). Megakaryocytic BP as initial manifestation of CML is even more rare, with few cases reported in the literature (Pullarkat S. Leuk Res. 2008 Nov;32(11):1770-5). CML-BP (including those with megakaryocytic transformation), carries a dismal prognosis, and successful therapeutic approach to this group of patients remains a clinical challenge (Kantarjian H, Blood. 2002;99:3547-3553). *Aim.* To evaluate the natural history, clinical characteristics, response to treatment and outcome of patients with megakaryocytic BP CML. *Methods.* Since 1965, 16 patients with the megakaryocytic BP were treated at MDACC (only 2 since 2000): 5 as initial presentation of CML and 11 as transformations of previous CML. Their medical records were reviewed to define their clinical, laboratory, and pathologic characteristics, as well as treatment and outcome. *Results.* Megakaryocytic BP represented 1.3% of all BP seen during this period. Median age of presentation was 50 years (range, 30 to 67). Splenomegaly ≥5 cm bellow costal margin was seen in 9 patients; 4 had extramedullary presentation in (bone, gastrointestinal tract, central nervous system, and subcutaneous, respectively); 8 had hemoglobin ≤10 g/L. The median WBC was 16.3 $\times 10^9$/L (range,

3.8 to 100), platelets 248×10⁹/L. (6 to 960). Bone marrow fibrosis ≥50% was seen in 7 patients; 9 had clonal evolution with wide range of complex chromosomal translocations. Molecular analysis was done on 3 patients who had 210kDa transcript detected, and only 1 patient had mutational analysis completed (NPM1 mutation detected, no abl kinase domain mutation). At the time of BP diagnosis, 2 patients received tyrosine kinase inhibitor (TKI)-based therapy (TKIs + chemotherapy), 6 received Ara-C + anthracyclins-based chemotherapy, and 8 had other forms of chemotherapy (including fludarabine, mitoxanthrone, decitabine, DHAD, and hydroxyurea). Subsequently, 7 patients received one, 6 patients received two and 3 patients received ≥3 salvage treatments. Fifteen patients were evaluable 4 weeks after the treatment: 6 had complete response, 9 had no response (including 2 early deaths). The median survival for the total population was 7 months, with 29% alive at 1 year, with no difference in outcome between patients with *de novo* presentation and those with transformation. *Conclusion.* Megakaryocytic BP of CML is a rare entity, with poor response to therapy and short median survival. Addition of TKI may improve the response.

0835

EFFICACY AND SAFETY PROFILE OF DASATINIB IN A SUBSET OF VERY ELDERLY PATIENS WITH CHRONIC MYELOID LEUKEMIA (CML) RESISTANT/INTOLERANT TO IMATINIB

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Dasatinib is a second generation TK inhibitor active in CML resistant and/or intolerant to imatinib. Data on efficacy and safety profile in "very elderly"patients (age >75 yrs) are scanty. We studied 28 patients with CP-CML treated with dasatinib when aged >75 yrs at 16 Centers. Median age at diagnosis was 72.3 yrs (IR 62.8-83.9). Male/female ratio was 1.33 (M=16, F=12); Sokal risk was calculated in 20/28 cases (low risk n=4, intermediate n=12, high n=4). Twenty-four patients (85.7%) received a treatment before imatinib. Imatinib starting dose was 400 mg/day in 21 cases (75%) and median period of therapy was of 36.6 months (IR 2.8-71.3). Cause of imatinib failure was primary resistance in 15 patients (53.6%), secondary resistance in 11 (39.3%) and intolerance in 2 (7.1%). Median age at dasatinib start was 79.3 years (IR 75.0-76.5), with a median time from diagnosis of 76.4 months (IR 4.7 - 173.9). Eleven patients (39.3%) have received a second-line treatment after imatinib stop. Baseline BCR/ABL mutational status was evaluated in 11 cases: 4 displayed no mutation; 3 cases had M244V; M315T, E494G, V299L were present in 1 case each; 1 case presented M351T and F317L. Twenty-three patients had at least one co-morbidity, and median number of concomitant medicaments was 3.5 (IR 1-11). Dasatinib was started at 140 mg/day in 12/28 cases (42.8%), 100 mg in 8 (28.6%), ≥50 mg in 8 (28.6%). After a median treatment period of 18.1 months (IR 0.9-39.8), grade 3-4 toxicity was evaluated: 5/28 patients (17.8%) showed hematological toxicity (140 mg n=2; 100 mg n=1; 50 mg n=2); 11 (39.3%) extra-hematological toxicity (140 mg n=6, 100 mg n=2, 50 mg n=3). Seven patients (25%) presented serosal effusions (SE): WHO grade 1-2 in 5 cases (17.8%; 4 cases 140 mg, 2 cases 50 mg); grade 3-4 in 2 (7.1%) (1 case 140 mg, 1 case 50 mg). Sixteen patients (57.1%) underwent dose reduction: of the patients treated with 140 mg (n=12), 11 had a dose reduction *vs.* 5 reductions in the 16 cases receiving ≤100 mg (P=0.005). Nine patients discontinued dasatinib for ≤6 weeks, while 7 (25%) permanently discontinued therapy: 3 (10.7%) due to drug-related toxicity (n=1 with 100 mg and gastrointestinal toxicity; n=2 with 50 mg and SE, grade 2 and grade 3 respectively), 4 (14.3%) for emergency of a T315I mutation, after a median of 11 months of treatment. Response to treatment was assessable in 24/28 patients (85.7%): 14 (53.8%) had a cytogenetic response (CCyR in 9 cases, PCyR in 3 and minCyR in 2); of these, 5 attained also a molecular response. Nine patients (57.5%) reached at least a complete hematological remission. One case (4.1%) was resistant. The mean Overall Survival and Event Free Survival of the whole cohort were 24.2 and 14.3 months respectively. The present analysis shows that dasatinib may have a major role in the treatment of very elderly patients resistant/intolerant to imatinib; moreover dasatinib 100 mg showed great efficacy and a favorable safety profile in pretreated patients.

0836

IMPROVING EQUITY FOR CML PATIENTS: DESIGN AND IMPLEMENTATION OF REAL-TIME QUANTITATIVE PCR (RQ-PCR) ASSAYS FOR MONITORING RARE VARIANT BCR-ABL1 FUSIONS, AND CHARACTERISATION OF A NOVEL BCR-ABL1 FUSION

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Molecular residual disease (MRD) monitoring using quantitative techniques is the standard of care for chronic myeloid leukemia (CML) patients and is recommended by the European LeukemiaNet (Baccarani M. *et al*. J Clin Oncol 2009; 27(35):6041-51). Approximately 98% of CML patients have common e13a2 or e14a2 (formerly b2a2 or b3a2) BCR-ABL1 gene fusions, with breakpoints in introns 13 or 14 of BCR, and intron 1 (1a or 1b) of ABL1. Standardised protocols for real-time quantitative RT-PCR (RQ-PCR) designed within the Europe Against Cancer (EAC) program are widely used to monitor these common BCR-ABL1 fusion types (Gabert J. *et al*. Leukemia. 2003; 17(12):2318-57). Around 2% of CML patients have rare BCR-ABL1 fusions with breakpoints outside the typical regions. Such patients are usually monitored using 'end-point' nested RT-PCR which, being non-quantitative, does not permit comparison of disease levels between sequential samples. This can preclude early detection of molecular relapse via increasing MRD levels, and impending treatment failure, resulting in an essentially second rate service for these patients. It was our aim to develop RQ-PCR assays for every rare variant BCR-ABL1 fusion type encountered by our laboratory. We characterised rare BCR-ABL1 fusions detected by RT-PCR in thirteen of our CML patients by sequencing the variant transcripts. This enabled us to design RQ-PCR assays based on modified EAC protocols. Now validated, these assays are being used to provide a clinical service, meaning that every CML patient (n=>500) within our region has access to quantitative molecular monitoring. Twelve patients have rare variant BCR-ABL1 fusions that have been reported previously (e13a3 n=6, e14a3 n=5, e19a2 n=1). The final patient has a variant of a typical e13a2 fusion, with a rare exonic break in exon 13 which has the effect of removing the standard EAC RQ-PCR forward primer binding site. Full characterisation of this apparently novel BCR-ABL1 variant is presented. None of these rare variant fusions can be detected by the standard EAC RQ-PCR protocol, and would thus be missed if genetic diagnosis of CML is based on standard RQ-PCR alone.

0837

PROGNOSTIC SIGNIFICANCE OF HOCT1 GENE EXPRESSION IN CML PATIENTS IN CHRONIC PHASE TREATED WITH IMATINIB

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Background. The human organic cationic transporter 1 (hOCT1) is a membrane protein which actively transports imatinib (IM) into the CML cells. It was shown that its high expression and activity in mononuclear cells is associated with higher probability of major and complete cytogenetic response (CCyR) and major molecular response (MMR), while low expression could be the cause of resistance to standard dose of IM. *Aim.* To evaluate the prognostic significance of hOCT1 expression in patients with CML-CP treated with imatinib. *Methods.* The expression of hOCT1 gene was measured by RQ-PCR in WBC obtained from 55 patients with CML-CP after informed consent was signed (45 pts resistant to standard dose of IM and 10 who achieved complete molecular response (CMR). hOCT1 expression was defined according to Wang LH *et al.* with the baseline of 181 arbitrary units

(a.u.) with the level of hOCT1 expression in CEM VBL 100 cell line kindly provided by Dr Wiliam T. Beck arbitrally defined as 1,0 a,u. In IM resistant patients direct sequencing was used to detect ABL kinase domain point mutation. The treatment failure, CHR, CCyR, MMR and CMR were defined according to the ELN guidelines criteria (Baccarani, J.Clin.Oncol., 2010). *Results.* In IM resistant pts the hOCT-1 expression (0,09-179, median: 63 a.u.) was low in 38% (17/45). 17 out of 28 resistant patients with high hOCT1 expression (191-23010, median: 596 a.u.) were available for mutation analysis. ABL mutations were detected in 4 patients (23,5%; 4/17) (2 mutations resistant to IM; 1 - sensitive to IM; 1 - sensitivity unknown). The expression of hOCT1 was high (516-1045, median: 617 a.u.) in all 10 patients who achieved CMR. Within the group of patients with high (>181 a.u) hOCT1 gene expression the difference between number of patients who achieved CMR and no responders was not statistically significant (P=0,8). Within the group of patients with low (<181 a.u) hOCT1 gene expression the difference between number of patients who did not respond to IM and achieved CMR was statistically significant (P=0,025). Patients with resistant mutations to IM were excluded from the analysis. Low hOCT1 expression seemed to be the cause of resistance to standard dose of imatinib in 28,9% of patients (13/45) as no other resistance predictor was identified. The probable cause of treatment failure in 2 patients with high hOCT1 expression was the presence of ABL gene point mutation resistant to imatinib. In remaining patients with high hOCT1 expression the mechanism of resistance was unknown. Expression of hOCT1 was high in all patients with CMR. *Conclusions.* hOCT1 gene expression analysis using RQ-PCR is convenient and clinically available. Our data suggest that the analysis of hOCT-1 gene expression more likely identifies poor responders to IM (with low hOCT expression); however if it is high the method seems to be insufficient to identify good responders.

0838

IMATINIB IN THE FIRST LINE CHRONIC MYELOID LEUKEMIA (CML) TREATMENT. CAN WE COMPARE THE REAL-LIFE DATA TO CLINICAL TRIAL RESULTS?

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Background. Imatinib (IM) substantially changed management of the CML and dramatically improved its prognosis, particularly in patients diagnosed in chronic phase (CP). The most robust source of data about the efficacy of IM in the first line CP-CML treatment is the IRIS trial. The data from real-life are still scarce. *Aims.* To evaluate the efficacy of IM in the first line treatment of a consecutive unselected population of CP-CML patients and to compare the real-life data to those obtained in clinical trials. *Methods.* We analyzed data about the 1st CP-CML patients from the defined region collected into the database called INFINITY (tyrosine kinase Inhibitors iN the FIrst aNd followIng CML Treatment). The evaluation of treatment responses was based on the valid definitions and recommendations according to European LeukemiaNet. We assessed rates and the cumulative incidences of complete hematologic responses (CHR), complete cytogenetic responses (CCyR), major (MMR) and complete molecular responses (CMR), and a comprehensive set of time-to-end points: overall (OS), transformation-free (TFS), progression-free (PFS), where progression was defined as in the IRIS, event-free (EFS), where also loss of CCyR, failure to achieve of particular response at the defined time-point and IM discontinuation due to toxicity were included, and our original point: an alternative treatment-free survival (ATFS), reflecting the real proportion of patients staying on IM despite of event. Among others we evaluated also tolerability of IM. *Results.* A total of 152 patients (median age 55 years, 20-77; 69 males and 83 females) treated with IM between 2003 and 2009 underwent the analysis. The median follow-up on IM treatment was 31.2 months (5.7-68.1). At 4 years, the cumulative incidences of CHR, CCyR, MMR and CMR were 95.3%, 80.6%, 65.4% and 39.2%, respectively. Overall response rates were as follows: 95.4% of CHR, 74.3% of CCyR, 57.2% of MMR and 24.3% of CMR. Estimated OS, TFS, PFS, EFS and ATFS at 4 years were 91.5%, 88.4%, 78.1%, 60.7% and 67.6%, respectively. In total, non-hematological and hematological toxicities of all grades occurred in 78.3% and 62.5%, respectively. However, in a majority of cases it was mild to moderate, with only 8.5% and 12.5% of gr. 3/4 of non-hematological and hematological toxicities, respectively. In total, 36 patients (23.7%) permanently discontinued imatinib after a median of 16.1 months (1-51) from various reasons: allogeneic stem cells transplantation (alloSCT) (n=4), IM intolerance (n=9), failure (n=7), failure + intolerance (n=1), progression (n=7), progression + failure (n=8). In total, 35 (23%) patients were given on alternative treatment, mainly on dasatinib (n=21). There were 7 deaths in total (4.6%); 3 patients died due to CML progression. Summary. We confirmed very good efficacy and tolerability of IM also in the cohort of patients treated outside of clinical trials in experienced specialized centers. However, with more appropriate definition of events in calculation of EFS, the overestimation of time-to-event analysis in IRIS trial is obvious.

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0839

EFFICACY OF IMATINIB MESYLATE ON CHRONIC PHASE CML PATIENTS ACCRUED IN THE SCREEN MULTICENTER STUDY: INTERIM REPORT ON THE FIRST 193 CASES

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Background and Aims. Imatinib mesylate (IM) has shown unprecedented effectiveness for the treatment of Chronic Myeloid Leukemia (CML) patients (pts) in the chronic phase of the disease. However, while most individuals achieve an optimal response to IM therapy, approximately 20% either fail IM or obtain an unsatisfactory suboptimal response. We analyzed the outcomes of all CML pts accrued to the observational SCREEN (Sicily and Calabria CML REgional ENterprise) multicenter study to evaluate the hematological, cytogenetic and molecular responses of this unselected population to IM according to the 2006 European Leukemia Net criteria. Patients and *Methods.* Although the study is still ongoing, 193 consecutive CML pts, after informed consent, have been enrolled in approximately five years by all the institutions involved. Each center was responsible for the diagnosis, treatment (IM 400 mg qd) and follow-up of the recruited pts, while all molecular analyses were centralized in Catania. Median follow-up time was 22 months. Pts characteristics were as follows: Age (median yrs): 54 (range 24-90); Sex (M/F): 108/85; WBC×10^9/L (median): 64.8 (range 3.4-718.0); Hb (g/dL): 12.1 (range 7.5-17.0); PLT×10^9/L (median): 330.0 (range 67.0-1690.0); Organomegaly %: 58; Sokal risk stratification %: 49 Low / 36 Intermediate / 15 High; Ph+ (mean % diagnosis): 96; ACA (% diagnosis): 7; BCR-ABL transcript variants %: 38, e13a2 / 53, e14a2 / 18, other; BCR-ABL IS % (median at Dx): 58.454. *Results.* Interim analysis was performed on an Intention to Treat basis. Currently 15 pts (7.7%) are off study. Cumulative incidences of complete hematologic response (CHR) and complete cytogenetic response (CCyR) were 98.3% and 84%, respectively. 58% of pts who obtained a CCyR also achieved a major molecular response (MMR). According to the ELN criteria, 121 pts (62.6%) presented an optimal response to IM; 36 pts (18.6%) had a suboptimal response (9 because of failure to achieve a CCyR by 12 months of therapy and the remaining 27 because of lack of a MMR after 18 months); 32 pts (16.5%) presented resistance to IM, because of either primary (20 pts) or secondary (12 pts) resistance. Only 4 pts were intolerant to IM. Interestingly, the median amount of BCR-ABL transcript at diagnosis (measured according to the International standardized Scale) displayed by pts that failed IM or achieved a suboptimal response (105.2 IS) was significantly higher than that of pts obtaining an optimal response (53.4 IS; P<0.001). *Conclusions.* In conclusion, IM was a highly effective and well-tolerated treatment for most chronic phase CML pts, producing high rates of CHR, CCyR and MMR. However, 35% of patients treated with IM either failed the drug or attained only partially satisfying results (suboptimal response). High levels of BCR-ABL transcript at diagnosis might allow a rapid identification of this less responsive CML patient population.

Drug resistance and pharmacology

0840

CONCOMITANT BCRP OVER-EXPRESSION AND FLT3-ITD MUTATION IDENTIFY A SUBSET OF ACUTE MYELOID LEUKEMIA PATIENTS AT HIGH RISK OF RELAPSE

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Background. Over-expression of different multidrug resistance (MDR) - related proteins in acute myeloid leukemia (AML) cells is associated with clinical resistance to chemotherapy and, consequently, treatment failure. Among MDR proteins, breast cancer resistance protein (BCRP, or ABCG2) is associated with a worse prognosis in AML patients. Another well-known prognostic factors is mutation of FLT3 gene, as patients with internal tandem duplication (ITD) mutation display a worse outcome. *Aims.* Test the role of BCRP and FLT3 on disease outcome and the correlations between these two factors. *Methods.* We analysed 118 cases of adult AML patients, homogeneously treated with a fludarabine-based induction therapy and two consolidation courses with cytarabine and idarubicine. Results. BCRP was over-expressed in 56/118 (47%) patients, and FLT3-ITD mutation was found in 33/118 (28%) cases. A significant correlation was found between BCRP positivity and FLT3 mutation, with 23 ITD in 56 BCRP-positive cases (41%) compared to 10 ITD in 62 BRCP-negative patients (16%) (P=0.004). After induction therapy, 78 patients (66%) attained a complete remission (CR). CR rate was negatively affected by FLT3-ITD mutation (P=0.02) but not by BCRP expression (P=0.13). Conversely, BCRP status had a strong impact on disease-free survival (DFS), with 18 relapses in 41 $BCRP^+$ cases (44%) and 8 relapses in 37 BCRP- patients (22%) (P=0.03). FLT3 mutation did not have an impact on relapse rate (P=0.14) when considered alone, but its negative effect on DFS was additive to BCRP over-expression, as $BCRP^+/FLT3^+$ patients had a highest relapse rate (10/19, 53%), compared to $BCRP^+/FLT3^-$ (8/22, 36%) and BCRP- cases, independently of FLT3 status (8/37, 22%) (P=0.04). Overall survival (OS) was affected by BCRP status (P=0.04), but not by FLT3 mutation (P=0.6). FLT3-ITD positivity did not significantly impact on OS in $BCRP^+$ patients, but this can be due to the high transplant rate in our population (70/118 patients were transplanted, 60%). BCRP and FLT3-ITD have a different impact on AML prognosis, as the first negatively affects DFS while the latter reduces CR rates, but the two factors are often co-expressed and $BCRP^+/FLT3^+$ patients display a significantly higher relapse rate and shorter DFS, with FLT3 mutation having an additive effect in $BCRP^+$ cases. Summary. In conclusion, in our study the co-expression of high BCRP levels and FLT-ITD mutation identifies a subgroup of AML patients with worse prognosis, that can beneficiate of an aggressive, post-consolidation therapy (i.e. allogeneic transplantation).

0841

A MECHANISM FOR INDUCTION OF APOPTOSIS FOLLOWING SHORT-TERM TYROSINE KINASE INHIBITOR (TKI) TREATMENT: INTRACELLULAR RETENTION OF TKI RESULTS IN PROLONGED CELLULAR TKI EXPOSURE AND INHIBITION OF KEY SIGNALING PATHWAYS

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Background. Small molecule tyrosine kinase inhibitors (TKI) have become a valuable tool in treatment of malignant disease. It has been widely accepted that continuous target inhibition *in vivo* is a prerequisite for successful TKI treatment. Surprisingly, administration of dasatinib once daily is clinically efficacious in CML despite transient target inhibition only and reappearance of BCR-ABL kinase activity after 24h. Recent data demonstrated that apoptosis is induced in BCR-ABL positive cells upon 2-4h incubation using dasatinib, whereas standard concentrations of imatinib were not effective in this setting. However, if high doses of imatinib are applied, one can also observe apoptosis upon short-term exposure. So far, the underlying molecular mechanism is unclear. *Aims.* Our study aims to elucidate the underlying molecular mechanism of induction of apoptosis upon short-term high-dose (HD) TKI treatment in hematopoietic cells dependent on oncogenic kinases. *Methods.* We employed cellular reconstitution models of hematopoietic Ba/F3 cells stably transfected with p190-BCR-ABL, and FLT3-ITD, respectively. Cells were incubated either with dasatinib or imatinib (BCR-ABL inhibition) or with PKC412 (FLT3-ITD inhibition) at three different dose levels (IC_{50}, IC80, HD) for 2-24h, followed by thorough washing steps. Cells were then incubated in medium without TKI. Upon a total incubation period of 24h, apoptosis was measured using flow cytometry and by Western blotting (caspase 3 cleavage). In parallel, inhibition of intracellular signaling pathways (pSTAT5, pERK) was determined by flow cytometry and Western blotting. To test for cellular retention of TKI, untreated cells were incubated with cell culture supernatant obtained after re-incubation of TKI treated cells for 2h in TKI-free medium. Again, apoptosis was measured at 24h and inhibition of signaling pathways was analyzed at various time points. Drug levels of imatinib in the cell culture supernatants before and after the washing procedures were determined by HPLC. *Results.* At the IC_{50} and IC80 TKI dose levels, a minimum incubation period of 16h was necessary to effectively induce apoptosis at 24h. However, using HD TKI (100 x IC_{50}) exposure for a period of 2h only was sufficient for induction of apoptosis. To test for possible cellular retention upon HD TKI treatment, we introduced additional washing steps and applied the supernatants to previously untreated cells. This analysis revealed: 1) Cells were completely rescued from apoptosis when additional washing steps were applied. 2) Supernatants induced high levels of apoptosis in previously untreated cells. To confirm that the nature of this effect is indeed cellular TKI retention, imatinib concentrations in cellular supernatants were monitored at various time points (0, 30, 60, and 120 min). This revealed a time-dependent dramatic increase in imatinib concentrations: 0 min: <0.2 μM; 30min: 0.71 μM, 60 min: 0.77 μM, 120 min: 0.82μM, while the IC_{50} has been determined as 0.25 μM in our assays. Additional studies employing imatinib plus PKC412 demonstrated that inhibition of MDR proteins is not responsible for this phenomenon. Summary/Conclusions. Together, our data derived from two tyrosine-kinase-mutant driven cell-models suggest that cellular TKI retention and consecutively prolonged inhibition of signaltransduction is the underlying molecular mechanism in short term HD TKI treatment.

0842

APOPTOTIC EFFECTS OF RESVERATROL ON IMATINIB SENSITIVE AND RESISTANT K562 CHRONIC MYELOID LEUKEMIA CELLS

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Background. Chronic myelogenous leukemia (CML) is a white blood cell disorder characterized by high levels of immature white blood cells. CML results from translocation between chromosome 9 and 22 that generates BCR/ABL fusion protein. BCR/ABL protein has constitutive tyrosine kinase activity which has important roles in cell growth, differentiation and evasion of apoptosis. Imatinib is the first target specific chemotherapeutic agent that specifically binds to the ATP binding pocket of the BCR/ABL. However, as seen in several cases, resistance to imatinib is the major problem of CML patients. Resveratrol is a naturally produced phytoalexin that is mostly synthesized in red gapes. It has anti-oxidant, cardioprotective, anti-inflammatory, and more importantly anti-tumor activities. *Aims.* In this study, we aimed to examine antiproliferative and apoptotic effects of resveratrol on imatinib sensitive and imatinib resistant K562 chronic myeloid leukemia cells and determine the mechanisms of resveratrol-regulated cell death. *Methods.* $Philadelphia^+$ human K562 cells were exposed to step-wise increased concentrations of imatinib and 3 μM imatinib resistant cells were developed, and referred as K562/IMA-3. Antiproliferative effects of resveratrol were determined by XTT cell proliferation assay and IC_{50} values (drug concentration that inhibit cell proliferation 50% comparing to untreated controls) were calculated from cell proliferation graphics. Apoptotic effects of resveratrol on K562 and K562/IMA-3 cells were determined through changes in caspase-3 enzyme activity, loss of mitochondrial membrane potential, and apoptosis by caspase-3 colorimetric assay kit, JC-1 mitochondrial membrane potential detection kit, and Annexin V-FITC, respectively. On the other hand, expression profiles of BCR/ABL in response to resveratrol were analysed by RT-PCR. *Results.* IC_{50} value of resveratrol were calculated as 85 and 122 μM in K562 and K562/IMA-3 cells, respectively. Three different concentrations (10, 50 and 100 μM) were selected from cell proliferation plots for assessment of apoptotic activity of resveratrol. The results revealed that there were 1.91, 7.42 and 14.73-fold increases in loss of mitochondrial

membrane potential in 10, 50, and 100 μM resveratrol applied K562 cells comparing to untreated controls. The same concentrations of resveratrol resulted in 2.21, 3.30, and 7.65-fold increases in loss of mitochondrial membrane potential in K562/IMA-3 cells as compared to untreated K562/IMA-3 cells. Caspase-3 enzyme activity results showed that there were 1.04, 2.77, 4.8-fold increases in K562 and 1.02, 1.41, 3.46-fold in K562/IMA-3 cells increases in response to the same concentrations of resveratrol, respectively. Annexin-V apoptosis assay results validate both caspase-3 enzyme activity and mitocondrial membrane potential *Results.* There were 4 and 3.7-fold increases in apoptotic K562 and K562/IMA-3 cell populations in response to 100 μM resveratrol as compared to their untreated controls. RT-PCR results showed for the first time that resveratrol downregulated expression levels of oncogenic BCR/ABL gene in a dose-dependent manner in both imatinib sensitive and resistant K562 cells. *Summary/Conclusion*. Taking together all these results may suggest potential use of resveratrol in both from responding chronic phase CML and from patients with primary and/or acquired resistance to imatinib.

0843

A NOVEL AUTOMATED ASSAY FOR THE DETECTION OF BCR-ABL KINASE DOMAIN MUTATIONS

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Background. Chronic myelogenous leukemia (CML) is caused by a consistent genetic abnormality, termed the Philadelphia chromosome (Ph). Imatinib mesylate (IM), now used as a standard first-line treatment of CML patients. However, resistance develops in a significant proportion of cases, and is predominantly mediated by single point mutations within the BCR-ABL kinase domain. Second generation TKIs such as dasatinib and nilotinib represent viable alternatives for IM-resistant or intolerant CML patients. Each mutated BCR-ABL has different sensitivity to those TKIs. Thus, it is significantly important to detect early the existence of BCR-ABL mutations and their specificities in treating Ph+ leukemia. *Methods.* We have developed a novel automated method that has high sensitivity to detection for a few copies of mutation sequences. This method is composed of PCR amplification step and Tm (melting temperature) analysis step that uses a quenching probe. When a whole blood sample or a purified DNA sample reacts with reagents, PCR and Tm analysis automatically processed in the same tube, and whole procedure finishes in approximately one hour. As Tm value of mutation sequence is higher than that of normal one, it is easy to detect the existence of mutation from the Tm analysis data. We have constructed the probes for 14 mutations concerned for IM-resistance (M244V, G250E, Q252H, Y253F, Y253H, E255V, E255K, T315I, T315A, F317L, M351T, E355G, F359V, and H396R). Considering the clinical significance of T315I mutation, which renders resistance to all currently available TKIs, we refined this method to higher sensitivity for detecting T315I mutation. *Results.* First, we analyzed the sensitivity of this system on BCR-ABL. In dilution assays, the system consistently quantified the mutation in a population containing as few as 3.0% mutant. Moreover, for T315I, we successfully detected as few as 0.3% (30 copies from 10,000 copies) mutations by a higher-sensitive assay. Next, we examined the clinical samples. Each sample was also examined by direct sequencing in comparison to our method. Kinase domain mutations were identified in 24 of the 50 (48%) patients. Our automated analysis was enabled to detect mutations in 19 patients, including p-loop mutations (G250E: n=3; E255K: n= 5), IM-binding domain mutations (T315I: n=10), and an activation-loop mutation (H396R: n=1). And all the positive cases showed a concordance with the result of direct sequencing. On the other hand, 5 cases were detected just by direct sequencing, but all that cases were out of setting mutations (Q252E, V379I, S417F, E459K). Impressively, in one case, only higher-sensitivity assay could reveal T315I mutation, although it was detected as a wild type both by direct sequence and our usual method. It suggests that the higher-sensitive system could detect low amount of T315I mutation in the earlier stage of disease. *Conclusions.* This rapid and accurate detection of clinically significant mutations enables us to contribute to better clinical practice in treating Ph+ leukemia patients, such as in selecting alternative strategies of IM dose escalation, second generation TKIs, or allergenic stem cell transplantation.

0844

ANTI-ANGIOGENIC EFFECT OF RESVERATROL AND CURCUMIN THROUGH THE INHIBITION OF MICROVESSEL DENSITY, VEGF AND ITS RECEPTOR VEGFR-2 (FLK-1) IN EHRLICH ASCITES CARCINOMA-BEARING MICE

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Background. Large numbers of chemo-preventive phytochemicals (naturally occurring substances found in plants) have been shown to possess anticancer activities by targeting different aspects of cancer progression and development. Since angiogenesis is pre-requisite for the growth of solid tumors, vascular targeting has been explored as a potential strategy to suppress tumor growth and metastasis. *Aims.* The present study was carried out to investigate the anti-angiogenic effect of two phytochemicals, resveratrol or curcumin, when used alone or in combination with carboplatin in Ehrlich ascites carcinoma (EAC)-bearing Swiss mice. Microvessel density (MVD) was used to evaluate anti-angiogenic effect. The levels of vascular endothelial growth factor (VEGF) in plasma and its receptor VEGFR-2 (Flk-1) in tumor tissue were measured as a possible mechanism through which these agents have achieved their anti-angiogenic effect. *Methods.* Solid tumors were induced by intradermal injection of EAC cells (2.5×10^6 cells/mouse), at 2 sites bilaterally, on the lower ventral side of Swiss mice. These tumors were used to evaluate effects of resveratrol (10 mg/kg, i.p), or curcumin (10 mM, 100 μL/mouse, i.p.) as individual treatments or in combination with carboplatin (5 mg/kg, i.p.), on MVD, VEGF and VEGFR-2 (Flk-1). All treatments were started 24 hours after tumor cells inoculation. Immunohistochemistry was performed for assessment of MVD and evaluation of tissue VEGFR-2 (Flk-1). Blood samples were collected on EDTA through the orbital sinus using heparinized microcapillaries and plasma levels of VEGF were determined using ELISA. MVD, VEGF, and VEGFR-2 (Flk-1) were determined as a time course on days 7, 14, and 21 post-inoculation. *Results.* Individual treatments with resveratrol or curcumin, alone or combined with carboplatin, produced a significant reduction in MVD compared to control and carboplatin-treated groups. Monotherapy of resveratrol or curcumin as well as their combination with carboplatin produced a significant difference in % reduction of MVD from control as compared to carboplatin monotherapy (Figure 1). Resveratrol or curcumin and their combination with carboplatin significantly reduced plasma levels of VEGF compared to control and carboplatin-treated groups on day 7 post-inoculation. EACs in the control group showed a strong expression of VEGFR-2 (Flk-1). Individual treatment with resveratrol or curcumin could reduce the percentage of VEGFR-2 (Flk-1)-rich tumors to reach 42.9% and 28.6%, respectively. The co-administration of resveratrol or curcumin with carboplatin has produced a further reduction in the percentage of VEGFR-2 (Flk-1)-rich tumors to reach 28.6% and 14.3%, respectively. *Conclusions.* We conclude from the current study that the phytochemicals, resveratrol and curcumin, inhibited angiogenesis as demonstrated by the reduction of MVD by these agents. Resveratrol and curcumin proved to exert their anti-angiogenic effect by inhibition of VEGF and its receptor, VEGFR-2 (Flk-1). The results suggest the beneficial role of these phytochemicals as adjuvant to chemotherapy in the treatment of cancer.

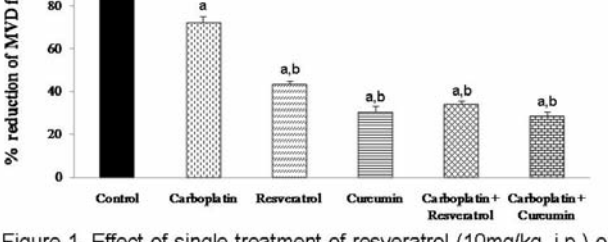

Figure 1. Effect of single treatment of resveratrol (10mg/kg, i.p.) or curcumin (10mM, 100μl/mouse, i.p.) and their combinations with carboplatin (5mg/kg, i.p.) on % reduction of MVD from control on day 21 post-inoculation in EAC-bearing male Swiss albino mice.

a: Significantly different from EAC-control at $p\leq0.05$
b: Significantly different from carboplatin at $p\leq0.05$

Figure 1.

0845

STAT5 IS A KEY MEDIATOR OF IMATINIB RESISTANCE IN ABELSON-INDUCED LEUKEMIA

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Background. In Bcr-Abl$^+$ leukemia, drug resistance is often associated with upregulation of Bcr-Abl or multidrug transporters as well as Bcr-Abl mutations. Nevertheless, many reportet cases of imatinib resistance still can not be explained with one of the known mechanisms. *Aims.* We want to find a new molecular factor responsible for reduced imatinb response of patients in late CML phases (accelerated and blast phases) and of patients with imatinib resistant chronic phase. The signal transducer and activator of transcription (Stat) family of transcription factors were promising screening candidates due to their reported implications in leukemias. *Methods.* We used primary Abelson (v-Abl, $p^{210Bcr\text{-}Abl}$ and $p185^{Bcr\text{-}Abl}$) transformed mouse cell lines harbouring different Stat5 protein levels due to gene dosage effects of Stat5 heterozygote cells or by overexpressing Stat5 using retroviral expression-vectors. To confirm our *in vitro* data, we could establish an *in vivo* model to evaluate imatinib sensitivity of leukemic cells in immuno-compromised NOD/SCID mice. To link our research data to the clinic, we analyzed Stat5 mRNA and protein level of several CML patients. *Results.* We show that the expression level of the transcription factor Stat5 is another parameter that determines the sensitivity of Bcr-Abl$^+$ cells against Bcr-Abl tyrosine kinase inhibitors (TKI) such as imatinib, nilotinib or dasatinib. Abelson-transformed cells expressing high levels of Stat5 were found to be significantly less sensitive to TKI-induced apoptosis *in vitro* and *in vivo*. This protection requires tyrosine-phosphorylation of Stat5 and transcriptional activity. In support of this concept, under imatinib treatment and with disease progression, Stat5 mRNA and protein levels increased in patients with Ph$^+$ chronic myeloid leukemia. *Conclusion.* Based on our data, we propose a model in which disease progression in Bcr-Abl$^+$ leukemia leads to an upregulated expression of Stat5, which subsequently confers protection against TKI-induced apoptosis. This process provokes the selection of cells with high Stat5-expression during treatment with TKIs and suggests that Stat5 may serve as a novel, attractive target to overcome imatinib resistance in Bcr-Abl$^+$ leukemia.

0846

A SINGLE DOSE, CROSSOVER, PLACEBO- AND MOXIFLOXACIN-CONTROLLED STUDY TO ASSESS THE EFFECTS OF BOSUTINIB (SKI-606) ON CARDIAC REPOLARIZATION IN HEALTHY ADULT SUBJECTS

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Background. Bosutinib, an orally bioavailable, small molecule, dual inhibitor of Src and Abl tyrosine kinases, is in phase 3 development for the treatment of patients with chronic myeloid leukemia (CML). An oral-dose of bosutinib 500 mg daily showed clinical efficacy in patients with Ph+ CML (Cortes *et al.*. Blood 112:1098, 2008). Tolerability and pharmacokinetic studies (Abbas *et al.*. Mol Cancer Ther 8:B215, 2009; Abbas *et al.*. Nature 85:S44, 2009; El Gaaloul *et al.* Eur J Cancer Suppl 7:132, 2009) demonstrated that single oral-doses of bosutinib 500 mg were well tolerated in healthy subjects, independently of increases in bosutinib exposures resulting from coadministration with food or ketoconazole (potent inhibitor of bosutinib metabolism that results in supratherapeutic exposures). Therefore, assessment of the effect of therapeutic and supratherapeutic bosutinib exposures on prolongation of cardiac repolarization, defined as prolongation of the corrected QT (QTc) interval, is feasible in healthy subjects. *Aims.* This study was conducted to assess the effects of therapeutic and supratherapeutic bosutinib concentrations on cardiac repolarization in healthy adults, in accordance with ICH E14 guidelines. *Methods.* This was a 2-part, randomized, single-dose, double-blind, crossover, placebo- and open-label moxifloxacin-controlled study in healthy subjects. In part 1, subjects received placebo, moxifloxacin 400 mg and bosutinib 500 mg with food (therapeutic dose). In part 2, subjects from part 1, following a washout, received placebo and bosutinib 500 mg coadministered with ketoconazole (supratherapeutic dose). Triplicate ECG readings were obtained. Repeated measures ANOVA was used to compare the baseline adjusted QTc interval for bosutinib with placebo (reference), and for bosutinib plus ketoconazole with placebo plus ketoconazole (reference) at each postdose time-point. The primary endpoint was QTcN (corrected QT based on a population-specific correction). Secondary endpoints based on Bazett's correction (QTcB), Fridericia's correction (QTcF), and individual-specific correction (QTcI) were also examined. PK/PD analyses and categorical summaries of interval data were performed. Linear regression models on change from baseline QTcN *vs.* log-transformed concentrations were fitted with postdose data for bosutinib and ketoconazole separately. Assay sensitivity was evaluated by the effect of moxifloxacin on the QTc interval compared with placebo. *Results.* Data for 56 healthy subjects (median age: 29 y; range: 18-50 y) were included in this study. Upper bounds of the 90% CI were <10 ms for the maximum mean change in QTcN from reference (4.57 and 6.82 ms) and at all postdose time-points for both the therapeutic and supratherapeutic bosutinib plasma concentrations, respectively, suggesting that therapeutic exposures (mean C_{max}, 114 ng/mL; mean AUC, 2330 ng$^-$h/mL) and supratherapeutic exposures (mean C_{max}, 326 ng/mL; mean AUC, 15,200 ng$^-$h/mL) of bosutinib were not associated with changes in QT interval. Results similar to QTcN were obtained for QTcB, QTcF and QTcI. No clinically relevant PK/PD relationship was observed between bosutinib concentrations and QTc interval. No subjects had a QTcB, QTcF, QTcI, or QTcN interval >450 ms or a change from baseline >30 ms. Moxifloxacin produced a significant increase in QTcN compared with placebo. Summary/*Conclusions.* This study demonstrated that therapeutic and supratherapeutic bosutinib exposures are not associated with QTc prolongation in healthy subjects.

0847

LIFE-THREATENING COMPLICATIONS DUE TO CLINICAL PHARMACOKINETIC INTERACTIONS BETWEEN CYCLOSPORINE A (CSA) AND TRIAZOLE ANTIFUNGAL AGENTS IN THE TRANSPLANTATION SETTINGS

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Background. It is well documented that the co-medication with the triazole antifungal agents is associated with a flattening of the CSA blood concentration profile via the cytochrome P450 3A4 dependent metabolic pathway in allogeneic hematopoietic stem cell transplantation (allo-HSCT). Then it raises the question if the therapeutic monitoring of CsA trough levels is sufficient enough to reflect the drug exposure and to assess the transplant-related complications such as transplant rejection and drug toxicity. *Aims.* To evaluate the tolerability, toxicity and clinical outcome of the co-administration of CSA and triazole antifungal agents in allo-HSCT recipients. *Methods.* A retrospective review of the medical records of 104 consecutive patients undergoing allo-HSCT for hematologic malignancies at our transplant center over past 5 years was conducted. The causality of administration of CSA in combination with triazole in 12 cases (11.54%) with graft rejection or life-threatening complications experiencing supratherapeutic trough levels of CSA were identified and analyzed. *Results.* Out of 12 patients, 5 patients received itraconazole prophylaxis, 3 voriconazole treatment, 4 itraconazole prophylaxis sequenced with voriconazole treatment. 2 cases developed acute graft rejection but still alive, in which CSA dosage and trough levels were not influenced by the presence of itraconazole prophylaxis. That indicates the trough plasma concentration might be inadequately reflect CSA absorption profile. In other 10 patients, the CSA trough levels remained highly than therapeutic range even after gradual tapering of CSA dosage. Shortly after the engraftment, 6 patients (50%) developed acute graft-versus-host disease (aGVHD) and, either idiopathic pneumonia syndrome (IPS) or diffuse alveolar hemorrhage (DAH); Whereas, 3 (25%) patients were evaluated with Progressive multi-focal leukoencephalopathy (PML), 1 (8.33%) PML accompanied by acute renal failure and liver dysfunction, that might be ascribed to CSA-related adverse effects. These 10 patients eventually died. The median survival time was 46.5 days and the major causes of death were non-infectious pulmonary complication or PML. *Conclusions.* Although preemptive CSA dosage reduction and the close monitoring of its whole blood trough levels may minimize the drug-toxicity when co-administration with triazole antifungal agents, the different clinical spectrum and different disease evolution post transplant in this group warn that in the settings of hematopoietic cell transplantation with CSA as immunosuppression agent the individual variables influence the drug-exposure and drug-toxicity. Thereof, as clinicians we should not be mis-

lead by trough plasma concentrations as a routine therapeutic drug monitoring in terms of CSA exposure related efficacy or toxicity, especially when CSA in combination with triazole.

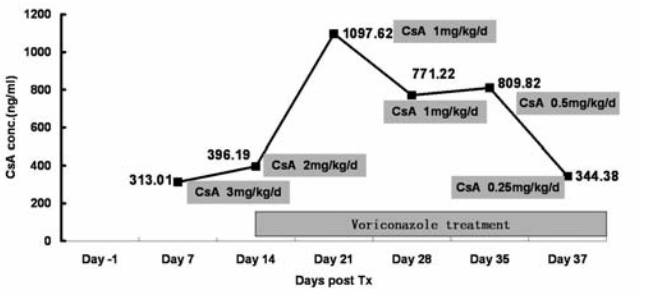

Figure 1. Example of individual pharmcokinetic interactions.

0848

VORICONAZOLE PHARMACOKINETICS DURING TREATMENT OR PROPHYLAXIS OF FUNGAL INFECTIONS IN IMMUNOCOMPROMIZED PEDIATRIC PATIENTS

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Background. Voriconazole is increasingly used for prophylaxis or treatment of fungal infections in patients treated for hematologic malignancies and/or recipients of allogeneic hematopoietic stem cell transplantation. In adults, voriconazole shows a non-linear pharmacokinetic profile, a narrow therapeutic window, drug-drug interactions and marked variability in plasma levels. Suggested voriconazole Ctrough concentrations for prophylaxis and treatment are reported to be >0.5 and >1.0 μg/mL, respectively. *Aims.* The study investigated voriconazole pharmacokinetics in a special population. *Methods.* 68 patients (44M, 24F) with hematological disorders, aged 1-23 years [16<7y;52≥7y], were enrolled. 36 patients received voriconazole for prophylaxis and 32 for treatment of aspergillosis. 21 patients were switched from OS to IV at different times during therapy. Voriconazole plasma samples were collected before and 2h after drug administration and levels measured on HPLC-UV. *Results.* We analyzed 304 Ctrough and 134 C2h plasma samples. An inverse correlation between D(mg/kg/12h)/Ctrough (mg/L) and age or BW was found. Patients under 7 years required higher doses/kg to obtain therapeutic levels (Table 1). Patients undergoing treatment received a median (range) dose/kg of 4.3mg (3.1-7.8) and 4.1mg (3.1-8.2) every 12h after OS and IV administration, respectively. Median Ctrough was 1.4 μg/mL (<0.1-4.6) and 2.2 μg/mL (<0.1-6.5) after OS and IV administration, respectively. Patients on prophylaxis received a median (range) dose/kg of 3.9 mg (1.47-6.13) and 4.51mg (1.82-6.73) every 12h, with OS and IV administration, respectively. Median Ctrough was 1.2 μg/mL (<0.1-5.1) and 1.3 μg/mL (<0.1-6.2) after OS and IV administration, respectively. Interpatient variability (CV%) in Ctrough levels was high, about 80%. Intrapatient variability was lower, that is 55%. Voriconazole oral bioavailability (F%) estimated in patients older than 7 years was 71%. *Conclusions.* Our data suggest the necessity to perform TDM in pediatric patients, to ensure a minimal level of exposure, with special consideration for children under 7.

Table 1. Pharmacokinetic parameters of voriconazole in the pediatric population.

Patients N.	Age (years)	Dosage (mg/kg/h)	C_{trough} (mg/L)	C_{2h} (mg/L)
Intravenous administration				
30	13 (7-18)	4.2 (2.2-6.6)	1.4 (<0.1-6.5)	
12	3.5 (1-5)	4.8 (3.1-6.0)	0.35 (<0.1-1.9)	
Oral administration				
25	15 (7-18)	3.9 (2.1-7.5)	0.9 (<0.1-2.9)	2.6 (0.1-5.2)
5	4 (3-6)	5.9 (3.6-8.0)	0.12 (<0.1-0.6)	1.26 (0.3-1.9)

0849

CYTOTOXIC EFFECT OF TETRACYCLINE ANALOGUES ON ACUTE MYELOID LEUKEMIA K562 CELLS

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Background. Despite extensive research to find appropriate treatment, still acute myeloid leukemia is one of the major causes of death among adults and children. Treatment related toxicity and poor outcome are the major limiting factors in cancer therapy. New effective mechanism-based treatment strategies with fewer side effects are warranted for further improvement. Tetracycline analogues (TCNAs) are used mainly as antimicrobial drugs. Recently, these drugs have been shown to possess cytostatic properties in several cell lines. Three major families of TCNAs have been identified, natural products, semisynthetic compounds and chemically modified tetracyclines. Doxycycline (DOXY) and minocycline (MINO) belong to semi-synthetic tetracyclines, while COL-3 belongs to the chemically modified tetracyclines that have no antibiotic properties. K562 is a myeloid cell line with M6 phenotypes and BCR/ABL fusion gene that is associated with resistance to cytostatic drugs. Effective therapy against this cell line might be potential treatment for cytostatic resistance leukemia. *Aim.* In this study, we investigated the cytotoxic effect of DOXY, MINO and COL-3 in human myeloid leukemia cell line K562. *Methods.* K562 cells were cultured in RPMI 1640 medium (supplemented with 10% FBS). Stock solutions of DOXY and MINO were prepared in sterile water, while COL-3 was dissolved in DMSO. Cells were treated (24-72h) with DOXY, MINO at concentrations of 10 to 200 μg/mL and with COL-3 at concentrations of 2.5 to 50 μg/mL. Control cells were incubated with either drug-free medium or DMSO (0.1%). Cell viability was evaluated using resazurin assay. Type of cell death was assessed by morphological criteria using May-Grünwald-Giemsa staining. Further confirmation of cell death type was carried out using Anexin V and propidium iodide staining and flow cytometry. Loss of mitochondrial membrane potential was investigated using tetramethylrhodamine methylester and flow cytometry. *Results.* All three drugs induced concentration-dependent decrease in viability of K562 cells at 24 h of incubation. The IC_{50} was 26, 20 and 21 μg/mL for DOXY, MINO and COL-3, respectively.Apoptotic and necrotic features were detected 6h after incubation with COL-3 (20 and 50 μg/mL) and after 48h at lesser concentrations. These features were observed after 24h of incubation with DOXY and MINO (50 μg/mL). No cytotoxic effect could be detected with DOXY and MINO at lower concentrations even after prolonged incubation-time. These results were in agreement with those found using Annexin V and propidium iodide staining. Loss of mitochondrial membrane potential started after 2h of incubation with COL-3 (20 and 50 μg/mL) and at 48h (2.5-10 μg/mL). However, the loss of mitochondrial membrane potential was first observed at 24h incubation with DOXY and MINO (50 μg/mL). No evidence of loss of mitochondrial membrane potential with DOXY and MINO at lower concentrations even with prolonged incubation, was observed. *Summary/Conclusion.* Cytotoxic effect of TCNAs on leukemic K562 cells was exposure dependent especially with COL-3. All three TCNAs induced apoptosis in the K562 myeloid cell line through mitochondrial-dependent pathway. COL-3 showed high anti-proliferative and pro-apoptotic effect compared to that observed for DOXY and MINO. Our results show that TCNAs may have treatment potential in acute myeloid leukemia.

0850

THE APOPTOTIC EFFECT OF BENDAMUSTINE COMBINED WITH CYTOSINE ARABINOSIDE (ARA-C) ON A MANTLE CELL LYMPHOMA CELL LINE

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Background. Bendamustine is a bifunctional agent that acts as an alkylating agent as well as a purine analogue with relevant clinical activity in mantle cell non-Hodgkin's lymphomas (MCL). Cytosine arabinoside (Ara-C) is widely used for treating young patients with MCL due to its recognized efficacy in this disease. Since these two agents exhibit individual and unique mechanisms of action in this tumor, a synergistic or additive effect might be expected when they are used in combination. *Aim.* Our aim was to investigate the effect of Bendamustine and Ara-C, alone and in combination, in modifying apoptotic pathways and cell proliferation of a MCL cell line model (JEKO-1). *Methods.* JEKO-1

cells were incubated with Bendamustine or Ara-C at concentrations ranging between 0 and 1000 µg/mL in RPMI medium supplemented with 10% fetal bovine serum, 2% L-glutamine and 1% penicillin/streptomycin. Drug dosages were chosen to reach the IC25, IC_{50}, IC75 and IC90 defined as the dosage of drug necessary to cause apoptosis of 25%, 50%, 75% and 90% of JEKO-1 cells, respectively. The two drugs were incubated simultaneously (24h and 48h, respectively) or consecutively (drug A or B for 2h, then washed and incubated for further 21h without any drug, then supplemented with drug B or A for 24h). Apoptosis was measured with the fluorescent DNA-binding agent 7-AAD (7 amino-actinomycin D), while cell proliferation/metabolic activity was measured using the tetrazolium-based assays, WST-1. Flow cytometric analysis was used for the first test, and the formazan dye (formed by the cleavage of tetrazolium salt in metabolic active cells) was quantified by a spectrophotometer. Results were expressed in terms of combination index (CI) which is a quantitative measure of the degree of drug interaction in terms of additive effect, synergism or antagonism. Calcusyn software was used to analyze results. *Results.* The cytotoxicity of Bendamustine alone was directly proportional to increasing drug concentrations, while Ara-C alone demonstrated a cytotoxic effect that was independent of the drug concentration. Noteworthy, the combination of Bendamustine and Ara-C was strongly synergistic on induction of apoptosis in all drug incubation schedules. In Figure 1 we show the CI values determined by apoptosis of all simultaneous and consecutive applied drug combinations. The highest CI (0.0076) was obtained with the incubation of Bendamustine followed by Ara-C (B+A in the higher two concentrations, as shown in Figure 1). Similarly, with the WST-1 test, we observed a stronger decrease in metabolic activity, which correlated to the cytotoxic effect of the drug, when Bendamustine and Ara-C were combined consecutively, even at low concentrations (data not shown). Discussion. Our data confirm the individual strong apoptotic effect of Bendamustine and Ara-C on JEKO-1 cells and reveal a considerable synergistic effect of the two drugs when used in combination, especially when Bendamustine was incubated before Ara-C. These findings give a rationale for the use of these two drugs in combination in the treatment of patients with MCL. We are now running a Phase II study with a regimen combining Rituximab, Bendamustine and Ara-C (R -BAC) in the treatment of MCL patients who are not candidate to high dose therapy.

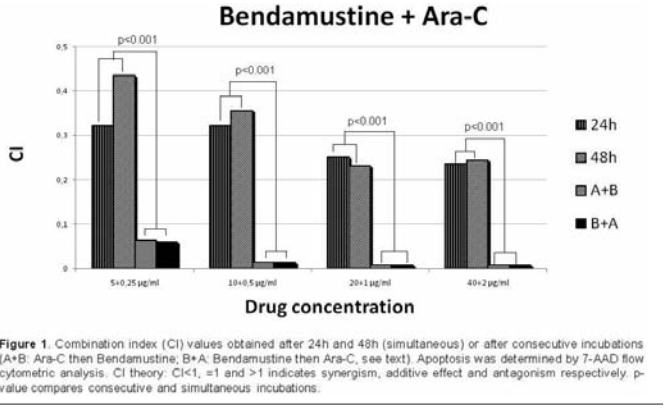

Figure 1. Combination index (CI) values obtained after 24h and 48h (simultaneous) or after consecutive incubations (A+B: Ara-C then Bendamustine; B+A: Bendamustine then Ara-C, see text). Apoptosis was determined by 7-AAD flow cytometric analysis. CI theory: CI<1, =1 and >1 indicates synergism, additive effect and antagonism respectively. p-value compares consecutive and simultaneous incubations.

Figure 1.

0851

SENSITIVE DETECTION OF E255K AND E255V P-LOOP MUTATION AND THEIR FREQUENCY AND CLINICAL SIGNIFICANCE IN IMATINIB RESISTANT CHRONIC MYELOID LEUKAEMIA PATIENTS

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Background. Despite durable responses to imatinib in chronic myeloid leukaemia (CML), mutations in Bcr-Abl kinase domain (KD) are known to induce imatinib resistance and cause poor clinical outcome.But the prevalence and prognostic significance of two P-loop E255K and E255V mutations in Indian patients. To shed light on the frequency, distribution, and prognostic significance of P-loop mutations this study was carried to identify a fast and sensitive method for screening E255K and E255V. *Aims.* To identify a fast and sensitive method for screening for E255K and E255V mutation in patients with imatinib-resistant chronic myeloid leukemia (CML). To study the frequency and clinical significance of E255K and E255V mutations in Imatinib resistant chronic myeloid leukemia patients. Patients and methods. The study included 80 imatinib resistant chronic myeloid leukemia patients registered during 2003-2009. DNA was isolated from the peripheral blood and Allele specific oligonucleotide polymerase chain reaction (ASO-PCR) assay was developed for the detection E255K and E255V mutations. Mutated and wild-type allele was specifically amplified in a 25ul reaction mixture . The incidence of mutation was correlated with different clinical and molecular parameters of the patient. The dosage of imatinib was 400mg to 800 /day. All patients were monitored for hematologic and molecular responses, time to progression, survival and toxicity. Results. All patients were monitored for hematologic and molecular responses and only resistant cases were selected and screened for E255K and E255V mutations. After a median follow-up of 3 years (range 3 to 5 years 60 (31.6%) patients had developed molecular relapse and hematological progression. The prevalence of E255K p-loop mutation was 50% (40/80) and of E255V p-loop mutation was 43.75% (34/80). Out of 34 E255V positive cases, 10 progressed to BC-CML and 10 responded to high dose Imatinib 800mg/day and three died. Out of 40 E255V positive cases, 08 progressed to BC-CML and 20 responded to high dose Imatinib 800mg/day. All were treated with imatinib at 600 to 800 mg/day. Survival and time-to-progression curves were obtained from Kaplan-Meier method. There was a significant difference in the survival rate of patients with or without E255V p-loop mutations. Patients with E255V p-loop mutations had short survival. Side-effects of imatinib included anemia (n=20), thrombocytopenia (n=19) and hypopigmentation of skin (n=10). The overall clinical outcome or prognostic significance of Imatinib resistant CML patients carrying E255V mutations was poor. Conclusions. Our findings suggest that the early detection of E255K and E255V mutations proved helpful in clinical management of therapeutic decisions. ASO-PCR is a very economical, sensitive and rapid technique for detection of E255K and E255V mutation and is more sensitive than mutation detection by sequencing. The fastness, simplicity, and sensitivity of ASO-PCR assays will be useful for routine monitoring of mutations, especially for frequently identified mutations in the developing countries where direct sequencing facilities are not available. The detection of E255V and E255K mutation was associated with imatinib resistance, and poor prognosis.

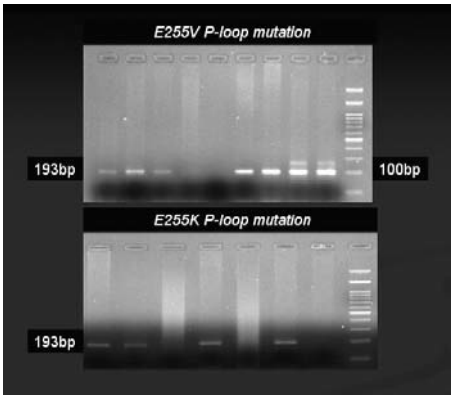

Figure. Gel picture of E255V mutation.

0852

A STUDY OF THE INFLUENCE OF TNF-ALPHA ON THE EFFICACY OF TREATMENT WITH RECOMBINANT HUMAN ERYTHROPOIETIN IN PATIENTS WITH MALIGNANCIES OF THE LYMPHOID SYSTEMS

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Background. Anemia in patients with malignancies of the lymphoid systems (MLS) frequently occurs and decreases the efficacy of antitumor therapy and survival of patients, also altering their quality of life. In such patients anemia is being corrected using transfusions of red blood cells and preparations of recombinant human erythropoietin (rHuEPO); rHuEPO significantly decrease volume of red blood cell transfusions and risk of complications. Nevertheless, it is not always possible to predict the rHuEPO treatment efficacy, while the cost of such treatment is high.

Aims. The goal of this study was to evaluate the efficacy of anemia correction using rHuEPO in patients with malignancies of the lymphoid systems and to study the influence of serum TNF-alpha level on the results of rHuEPO therapy. *Methods.* The studied group of patients with MLS (n=21) consisted of patients with chronic lymphocytic leukemia (n=5), indolent forms of Non-Hodgkin lymphoma (n=7) and multiple myeloma (n=9). Age varied from 49 to 80 years (63.6±8.2 years). Anemia with hemoglobin level below 10.0 g/dL was an indication for rHuEPO prescription. All patients have previously received not less than 3 courses of chemotherapy. Before rHuEPO treatment 3 patients received red blood cell transfusions, according to vital indications (Hb<6.5 g/dL), after which hemoglobin increased to 8.0 g/dL. Those patients, in whom there was observed hemorrhage, hemolysis, iron or B_{12} deficiency, were not included in this study. Treatment with recombinant human erythropoietin was performed subcutaneously using 150 ME per kilogram body weight three times a week (weekly dose was 450 ME/kg). Before prescription of rHuEPO there were measured initial indices of hemogram (hemoglobin level, hematocrit, red blood cell count, reticulocyte rHuEPO treatment, Hb level increased to 12.0 g/dL and its monthly increase was ≥1.0 g/dL. *Results.* Duration of rHuEPO treatment was in average 10.1±3.6 weeks (4-16 weeks). Positive response to rHuEPO was observed in 13 of 21 patients (61.9%). There was observed significant (P<0.01) increase of hemoglobin level, in average from 8.68±1.85 g/dL (4.6-10.0 g/dL) to 11.14±2.65 g/dL (6.7-14.7 g/dL), red blood cell count - from 2.78±0.74×10^{12}/L (1.30-3.50×10^{12}/L) to 3.50±0.93×10^{12}/L (2.08-4.78×10^{12}/L), hematocrit - from 26.8±6.3% (13.4-32.7%) to 34.9±6,8% (20.6-46.2%). Also there was observed increase of reticulocyte count from 28.1±10.9×10^{9}/L to 79.9±50.3×10^{9}/L. In the whole group of patients TNF-alpha level before prescription of rHuEPO varied from 3.2 to 484.3 pg/mL (50.2±110.5 pg/mL). Depending on TNF-alpha level patients were divided into two groups. First group consisted of 14 patients with level of TNF-alpha less than 15 pg/mL (from 3.2 to 13.2 pg/mL, average 8.6±3.3 pg/mL). In this group positive response to therapy with rHuEPO was reached in 13 patients. Second group consisted of 7 patients with level of TNF-alpha more than 15 pg/mL (from 19.2 to 484.3 pg/mL, average 133±167 pg/mL). In this group positive response to therapy with rHuEPO preparations was not observed in any of the patients. There was established a reverse correlation between TNF-alpha level and efficacy of rHuEPO treatment (r=-0.487; P<0.03; n=21). Thus, in those cases when TNF-alpha was higher than 15 pg/mL, there could not be reached a positive response. *Conclusions.* Thereby, we have revealed that initial serum level of TNF-alpha in patients with MLS affects the efficacy of correction of anemia by rHuEPO, and its evaluation can be used as a predictor of response to rHuEPO treatment. Low level of serum TNF-alpha (<15 pg/mL) presumes a positive response to treatment, while high level (>15 pg/mL), as a rule, indicates resistance to rHuEPO treatment in such patients and non-expedience of prescription of stimulators of erythropoesis.

0853

ACTIVATION OF CYCLIC AMP SIGNALING INHIBITS CHEMOTHERAPEUTIC-INDUCED P53-MEDIATED APOPTOSIS IN WILD-TYPE P53 ALL CELLS

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Background. The p53 tumor suppressor protein is a potent roadblock to tumor development. Cells that are insulted by chemotherapeutic DNA-damaging agents or other forms of stress stabilize the p53 protein by phosphorylation or other modifications. Stabilized p53 accumulates in the nucleus to regulate the expression of numerous pro-apoptotic genes. We evaluated the effect of cAMP signaling pathway, an important regulator of hematopoietic cell proliferation, differentiation and apoptosis, on p53-mediated apoptosis in response to DNA-damaging agents. *Aims.* The aim of this study was to evaluate the effect of activation of cAMP signaling system on wild-type p53 leukemia cells upon exposure to chemotherapeutic drugs. In addition, we dissect the molecular mechanisms of cAMP action on p53 in order to find the main mediator of p53 destabilization. *Methods.* Cultured pre-B ALL NALM-6 cells and T-ALL MOLT-4 cells were exposed to doxorubicin in the presence or absence of cAMP-increasing agents for 24h and then cells were subjected to apoptosis analysis by flow cytometry. Western blot method was used to analyze phosphorylation state of p53 protein, total p53, and the levels of other proteins which were involved in doxorubicin-induced apoptosis. Real-time PCR was performed to analyze the expression levels of p53 target genes. cAMP specific analogs were used to find the mediator of cAMP action on p53 destabilization. In addition, RITA was used to show the role of HDM2, a physiological negative regulator of p53, in cAMP-induced p53 degradation. Results. Our results indicate that elevation of cAMP in B cell precursor acute lymphoblastic leukemia (BCP-ALL) cells and T-ALL MOLT-4 cells profoundly inhibit the apoptotic response to doxorubicin. We further demonstrate that this effect depends on the ability of elevated cAMP levels to quench DNA damage-induced p53 accumulation and phosphorylation. Increased cAMP levels also repressed the expression of proapoptotic p53 target genes. Using cAMP specific analogs we found that this inhibitory effect of cAMP is not mediated through PKA or EPAC which are the main mediators of cAMP-signaling system. The results of specific inhibitor of p53-HDM2 interaction showed this action of cAMP in not mediated through HDM2. *Summary/Conclusions.* In conclusion, recognition that elevated cAMP levels may abrogate p53-dependent apoptosis carries clinical implications for patients with wild-type p53 leukemia cells undergoing curative antineoplastic therapy, because a large number of common agents modulate cAMP metabolism.

0854

LABORATORY VALIDATION OF THE MULTIDRUGQUANT™ ASSAY KIT

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Background. The multidrug resistance usually results from the expression of ATP-binding cassette (ABC) transporters, such as the ABCB1 (MDR1 or P-gp), ABCC1 (MRP1), and ABCG2 (MXR or BCRP) which are known to function as drug efflux pumps. Although it is believed to be a major barrier to successful chemotherapy in cancer patients, neither the genetic polymorphisms nor the mRNA or protein expression levels correlate closely with the functional activity and studies using the methods above have given conflicting and inconsistent results. On the other hand, although the functional methods separately gave promising results, standardization and reproducibility of these tests failed to conform to the values required from routine diagnostic methods. MultiDrugQuant kit was developed an improved functional assay system, which can measure the multidrug resistance activity of the three, clinically most relevant efflux transporters, such as MDR1, MRP1 and BCRP in living tumor cells. *Aim.* Our purpose was the laboratory validation of the MultiDrugQuant-kit prior to the performance evaluation in haematological diseases. *Methods.* Validation of the kit was carried out according to the standards of the Clinical Laboratory Standards Institute in three university centres in Hungary. Mononuclear cells were separated using Ficoll gradient and tested at 2×'5×-10^{6}/mL within 6 hours after specimen collection. The kit applies fluorescent dyes (substrates) and inhibitors of the all three transporters implicated. The activity of the multidrug transporter was calculated from the difference between the mean fluorescent intensity of cells w/o the specific inhibitors, respectively. Inaccuracy and comparative measurements were carried out using cell lines with low and high activity of transporters. Results on different flow cytometers were compared using CD45 or CD19 or CD3 monoclonal antibodies for gating to detect the population of interest. *Results.* The assay proved to be specific and quite robust at 10-100% of the fluorescent dyes or 50-150% of the inhibitors. Both intraassay and interassay reproducibility were <5%. We found comparable and eligible multidrug resistance activity values, which were determined on different flow cytometers. *Conclusion.* The MDQ-kit provides quantitative results on the multidrug resistance activity of the MDR1, MRP1 and BCRP. Measurement of the activity of these membrane transporters in the target cells might be used to predict the resistance of these cells to particular cytotoxic agents. Recently, clinical trial was started in haematological diseases and we will follow up the therapeutic response and the correlation with the transporter activity in order to evaluate the predictive value of the biomarker.

0855

PRECLINICAL EVALUATION OF ANTITUMOR ACTIVITY OF BORTEZOMIB IN COMBINATION WITH EPIGALLOCATECHIN GALLATE (EGCG), A COMPONENT OF GREEN TEA

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Background. Bortezomib is a first in class proteasome inhibitor, currently approved in the United States and Europe for the treatment of patients with multiple myeloma (MM) and approved in the US for relapsed or refractory mantle cell lymphoma. The boronate group of bortezomib can chemically interact with 1,2 diol groups on polyphenols including epigallocatechin gallate (EGCG), a major component of green tea. Previously published studies in cultured cells indicate that high concentrations of EGCG (2.5 μM-10 μM) can antagonize the ability of 10 nM bortezomib to inhibit the proteasome and kill cells. However, it is unknown whether this antagonism would occur in patients receiving bortezomib treatment while consuming green tea or EGCG supplements. *Aims.* To evaluate whether EGCG can antagonize the antitumor effects of bortezomib *in vivo*, using the bortezomib-sensitive CWR22 prostate-derived xenograft tumor model grown in immunocompromised mice. *Methods.* We first characterized the pharmacokinetics (PK) of EGCG dosed orally, intravenously (IV), and subcutaneously (SC) in immunocompromised mice. Our goal was to find a dose and route of administration resulting in EGCG plasma concentrations in the range of those measured in human PK studies of EGCG dietary supplements, so that our preclinical studies would test clinically relevant plasma concentrations of EGCG. Published studies of EGCG in human plasma show maximal concentrations of approximately 0.4 μM and 3 μM when 800 mg supplements are taken with food or on an empty stomach, respectively. 800 mg of these supplements is equivalent to approximately 8 cups of brewed green tea. In mice, oral dosing of EGCG at 50 mg/kg resulted in very low plasma concentrations, with C_{max} of only 0.076 μM. SC and IV dosing of 50 mg/kg EGCG achieved much higher plasma concentrations (30 μM and above), and these routes were used in combination studies with bortezomib. *Results.* Bortezomib dosed IV at its twice-weekly maximum tolerated dose (0.8 mg/kg) in CWR22-xenograft bearing CB17-SCID mice results in a consistent antitumor response, with a ratio of average tumor volume in the treated *vs.* control group (T/C)=0.37-0.46. Twice-weekly dosing of 50 mg/kg EGCG by IV or SC administration had no antitumor activity on its own (T/C=0.8-1.15). In combination studies using SC dosing of EGCG and IV dosing of bortezomib, when EGCG plasma concentrations as high as 11-15 μM were present at the time of bortezomib dosing, no antagonism was detected (T/C=0.36-0.54). However, antagonism was detected in a combination study using IV dosing of EGCG and bortezomib, when the EGCG plasma concentration was 233 μM at the time of bortezomib dosing (T/C=1.18). This concentration of EGCG is more than 75 times higher than the maximal concentration of EGCG measured in humans taking 800 mg supplements while fasting. *Conclusions.* These results suggest that the level of EGCG required to antagonize bortezomib is not physiologically relevant in patients taking oral EGCG supplements or drinking green tea.

0856

SUPPRESSION OF STAT5A INCREASES CHEMOTHERAPEUTIC SENSITIVITY IN IMATINIB-RESISTANT AND IMATINIB-SENSITIVE K562 CELLS

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Background. STATs (Signal Transducers and Activator of Transcription Proteins) are latent cytoplasmic transcription factors that upon activation carry information coming from outside the cell into the nucleus. Extensive studies in mammalian systems revealed that STATs consist of seven structural and functional homolog family members. STAT activation is normally achieved by phosphorylation of specific tyrosine residues accomplished either by ligand-activated receptor-tyrosine kinases or receptors lacking intrinsic tyrosine kinase ability. Chronic myeloid leukemia (CML) is a hematological cancer that is characterized by the accumulation of immature leukemic cells of myeloid origin in the bone marrow and bloodstream. Main driving force of this malignancy is the reciprocal translocation between the q arms of the 9. and 22. chromosomes, bringing the BCR and ABL genes together. The resultant chromosomal structure is referred as the Philadelphia chromosome (Ph) and the whole translocation process results in the expression of the BCR/ABL fusion protein. Imatinib, a tyrosine kinase inhibitor, binds to the ATP binding domain of the fusion protein and prevents the subsequent phosphorylation of the target proteins. Survival periods of CML patients can substantially prolonged by means of this drug, but in most cases gained resistancy can affect the clinical outcome of this therapy. Resistancy, on the other hand, is conferred by various mechanisms. *Aims.* In this study we aimed to identify the differentially expression pattern of STAT genes in imatinib-sensitive and -resistant K562 CML cell lines; and further, to reveal the effects of STAT5A siRNA knockdown on cellular growth and apoptotic induction. *Methods.* mRNA expressions of STAT genes were assessed by quantitative real-time PCR (Q-PCR). For silencing the STAT5A gene in imatinib-sensitive and 3 μM imatinib-resistant K562 cell lines (K562/IMA-3), HiPerFect Transfection Reagent was used combination with cholesterol-conjugated anti-STAT5A siRNAs. Cell proliferation was detected by the XTT cell proliferation assay and apoptosis was evaluated by changes in Caspase-3 enzyme activity. *Results.* Q-PCR analysis revealed that the expression level of STAT5A most significantly changed among the other STATs examined in K562-IMA3 cells compared to imatinib-sensitive K562 cells. Its expression increased by 67%, whereas the expressions of STAT5B and STAT3 were increasing 56% and 4%, respectively. Our results demonstrated that silencing of STAT5A sensitized both sensitive and resistant K562 cells to imatinib. Transfected resistant cells became almost 4.5-times more sensitive than the non-transfected counterparts, while transfected sensitive cells showed approximately 1.12-fold increased sensitivity. These results indicate that imatinib-resistant K562 cells are more effectively sensitized by silencing of STAT5A compared to imatinib-sensitive K562 cells. For both sensitive and resistant cells, enhanced apoptotic induction was concurrent with suppression of STAT5A expression. Non-transfected K562/IMA-3 cells showed 1.85- and 3.46-fold increases in Caspase-3 enzyme activation in the presence of 5- and 10 μM of imatinib, while siRNA-transfected counterparts showed 11.21- and 20.17-fold increases. Conclusion. Observing these significant responses to imatinib after transfection with single gene-specific siRNAs might provide new opportunities for dealing with the frequent occurrence of resistance to chemotherapeutic agents in leukemia.

0857

MUTATIONS IN KIT PREDICT RESISTANCE TO SEVERAL TKI (SORAFENIB, SUNITINIB, AND MASATINIB) IN NEOPLASTIC HUMAN AND CANINE MAST CELL LINES

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Advanced mast cell (MC) disorders are characterized by uncontrolled growth of neoplastic MC in various organs, resistance against cytoreductive drugs, and a poor prognosis. In most patients, transforming mutations in the KIT proto-oncogene are detectable and are considered to contribute to resistance. MC lines are an important model for analyzing drug resistance. We have established a novel canine mastocytoma cell line, NI-1 from a canine patient suffering from mast cell leukemia. NI-1 cells were found to harbour several homozygous KIT mutations including two single nucleotide mutations, one at nucleotide 107 (C to T, leading to a missense mutation, P36L) and another at 1187 (A to G, leading to a missense mutation, Q396R), a 12 bp duplication at nucleotide 1263, and a 12 bp deletion at nucleotide 1550, the latter reflecting a transcriptional variant. Drug response profiling revealed that NI-1 cells differ markedly from canine mastocytoma C2 cells harbouring an exon 11 KIT mutation, and the human mast cell lines HMC-1.1 (exon 11 mutation in KIT) and HMC-1.2 (exon 11 and exon 17 mutation). In contrast to C2 cells, NI-1 cells were found to be largely resistant against the apoptosis-inducing effects of masatinib, sorafenib, and sunitinib. Similarly, in contrast to HMC-1.1 cells, HMC-1.2 cells were found to be largely resistant against masatinib, sorafenib, and sunitinib. Drug resistance was specific for KIT tyrosine kinase blockers in that vorinostat, an HDAC inhibitor, produced growth-inhibitory and apoptosis-inducing effects with comparable IC_{50} and ED50 values in all MC

lines tested. Together, these data provide further evidence that KIT mutations are associated with resistance to KIT-targeting drugs. The novel MC line NI-1 may serve as a valuable tool to investigate the mechanisms of drug resistance in canine mast cell tumors and the role of novel KIT mutations.

0858

THE EFFECT OF FOOD ON THE PHARMACOKINETICS OF ANAGRELIDE AND ITS ACTIVE METABOLITE 3-HYDROXYANAGRELIDE

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Background. Anagrelide, a novel imidazoquinalone, reduces platelet counts in humans and is licensed for the treatment of essential thrombocythemia. Since the active metabolite, 3-hydroxyanagrelide, appears to be exclusively produced by CYP1A2, anagrelide metabolism may be influenced by normal dietary constituents such as caffeine. *Aims.* To compare the pharmacokinetics of anagrelide and 3-hydroxyanagrelide when anagrelide is administered in fed and fasted states. To assess the safety and tolerability of anagrelide. To measure individual levels of expression of CYP1A2 by measuring the plasma caffeine:caffeine metabolite (paraxanthine) ratio and relate this to observed exposure to 3-hydroxyanagrelide. *Methods.* This was an open-label, single-dose crossover study of healthy, adult volunteers. Subjects were randomized to receive a single oral dose of anagrelide 1 mg alone (fasted), or following a standardized high-fat meal, including 2 cups of coffee (fed/caffeine), and crossed over to the other treatment after an interval of ≥3 days. Plasma concentrations of anagrelide and 3-hydroxyanagrelide were monitored for 24 hours after dosing. Adverse events (AEs) were recorded throughout the study. Caffeine:paraxanthine plasma ratios were measured 4-hours post-dose. *Results.* In total, 35 subjects (20 males, 15 females) entered the study, and 34 completed both the fasted-fed/caffeine and fed/caffeine-fasted treatment sequences. One female subject discontinued for personal reasons. Pharmacokinetic data are summarized in Table 1. There were significant differences between fed/caffeine and fasted states for both anagrelide and 3-hydroxyanagrelide for t_{max} and t_{lag}. In addition the C_{max}, $AUC_{0-\infty}$ and $t_{1/2abs}$ were non-equivalent between fed/caffeine and fasted states for anagrelide and 3-hydroxyanagrelide. There were no marked differences in pharmacokinetic parameters between genders. AEs were reported by a similar number of subjects in the fasting (23 [66%] subjects) and fed states (19 [56%] subjects). The most common AE observed during the study was headache, recorded in 19 fasted subjects (54%) *vs.* 8 subjects (23%) following food/caffeine. The next most common AE reported were palpitations, all of mild intensity, involving 6 fasted subjects (17%) and 12 (35%) following food/caffeine. ECGs were captured at the time of the events in a majority of subjects and were deemed to be normal. Transient rises in heart rate were observed at the time of the event. Measurement of the caffeine:paraxanthine plasma ratio following ingestion of two cups of coffee did not provide any evidence for a possible relationship between exposure to 3-hydroxyanagrelide and CYP1A2 status.

Table 1.

Arithmetic mean pharmacokinetic parameters for anagrelide and 3-hydroxyanagrelide (fasted and fed)

	Median t_{max} (h)	C_{max} (ng/mL)	AUC_{last} (ng.h/mL)	$AUC_{0-\infty}$ (ng.h/mL)	$t_{1/2z}$ (h)	t_{lag} (h)	$t_{1/2abs}$ (h)
Anagrelide							
Fasted	**1.50**	**5.1**	**12.9**	**13.1**	**1.7**	**0.34**	**0.43**
Fed	**4.00**	**4.4**	**15.2**	**15.9**	**1.8**	**1.34**	**0.74**
3-hydroxyanagrelide							
Fasted	**1.25**	**8.3**	**28.2**	**29.2**	**3.1**	**0.33**	**0.78**
Fed	**4.00**	**5.9**	**27.9**	**29.7**	**3.3**	**1.01**	**1.02**

t_{max} – time of maximum observed plasma concentration, C_{max} – maximum observed plasma concentration, AUC_{last} – area under the plasma concentration-time curve up to the last quantifiable concentration, $AUC_{0-\infty}$ – total area under the concentration-time curve, $t_{1/2z}$ – apparent half-life of the terminal elimination phase, t_{lag} – lag time before the first quantifiable plasma concentration, $t_{1/2abs}$ – apparent half-life of the absorption phase

Conclusions. In both fed/caffeine and fasted states anagrelide was rapidly absorbed following a single oral dose. Anagrelide and 3-hydroxyanagrelide are eliminated rapidly, with no appreciable pharmacokinetic differences between genders. The main effects of food/caffeine were to delay t_{max} of anagrelide and 3-hydroxyanagrelide by approximately 3 hours and to lower the maximum concentration of 3-hydroxyanagrelide by 30%. However, total exposure to either compound was not affected by food/caffeine. AEs observed during the study were in line with those previously reported and were similar in the fed and fasted states. Caffeine inclusion in the standardized meal may have contributed to the slightly higher rate of palpitations experienced in the fed state.

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0859

GENERATION OF A DATABASE ON INTERACTION OF DRUGS WITH MDR ABC TRANSPORTERS

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Background. Since the beginning of the 90's several studies have confirmed the role of multidrug transporter proteins in chemoresistance in a variety of haematological malignancies. The studies focused on rapidly progressing acute leukaemia's, with the emphasis being on acute myeloid leukaemia (AML). To characterize drug - transporter interactions we developed two major forms of ABC transporter assays: whole cell-based assays and membrane assays. MultiDrugQuant™ Assay Kit was used to assess function of the clinically most important multidrug resistance transporters (ABCB1/MDR1, ABCC1/MRP1) in living tumor cells. BCRP (ABCG2/MXR) a protein also implicated in multidrug resistance was also tested. *Aims.* Aims of this study were to test the interactions several drugs and ABC efflux transporters, characterize the interaction and correlate membrane as well as cellular data using in silico passive permeability values. *Methods.* In our study we used *in vitro* membrane based methods (ATPase and vesicular transport assay) and cellular assays (Hoechst assay and calcein assay) to show interactions between the MDR1/MRP1/BCRP transporters and different chemotherapeutic (e.g. chlorambucil, cisplatin, taxane) and immunosuppressive drugs (e.g.NSAID, DMARD). ATPase and VT assays were performed on membrane preparations from recombinant baculovirus infected Sf9 cells. Cellular dye efflux assays (Calcein, Hoechst) were performed on transfected or selected cell lines overexpressing the implicated transporter as well as inhibitors (verapamil, KO134). *Results.* We had characterised substrate or inhibitory profile of 35 drugs. IC_{50} values (dye efflux, inhibition of vesicular transport and ATPase as well as EC50 values (ATPase activation) will be presented. Passive permeability greatly affects correlation between membrane and cellular data. In general, the lower the passive permeabilty is the greater the $IC_{50\ cell}/IC_{50\ membrane}$ ratio is. *Conclusion.* This database will be useful in personalized medicine. The toolkit presented here can be used in drug discovery and development of chemotherapeutics.

Infectious diseases and supportive care

0860

GALACTOMANNAN-GUIDED PRESUMPTIVE VS EMPIRICAL ANTIFUNGALS IN THE PERSISTENTLY FEBRILE NEUTROPENIC - A PROSPECTIVE RANDOMISED STUDY

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Background. Invasive mold infections (IMI) cause significant morbidity and mortality in neutropenic patients. The risk of IMI increases with the severity and duration of neutropenia. As a result of the difficulty in making a diagnosis of IMI, empiric antifungals for persistent febrile neutropenia has become the standard of care. However, the empirical use of antifungals may lead to over-treatment. A new strategy, "presumptive"antifungal therapy, has emerged as an alternative to empirical antifungals in the management of febrile neutropenia. *Aims.* To determine if serial galactomannan (GM) monitoring, with effective anti-candidal prophylaxis, can obviate the need for empirical broad-spectrum antifungal treatment in patients receiving intensive chemotherapy for hematological malignancies and allogeneic hematopoietic stem cell transplant (HSCT) recipients. *Methods.* This prospective, randomized, non-blinded, ethics committee-approved study was conducted from June 2006 to October 2007 in the Department of Hematology, Singapore General Hospital. Patients receiving intensive chemotherapy for hematologic malignancies where neutropenia is expected to last longer than 7 days and patients undergoing alloHSCT were randomized to either the monitoring (presumptive) arm or observational arm. Patients in the presumptive arm had regular GM assays and received caspofungin, amphotericin or voriconazole (CAV) for persistent febrile neutropenia(FN) if there were 2 positive GM results or a positive GM result and a CT thorax suggestive of invasive pulmonary aspergillosis (IPA). Patients in the observational arm received antifungals according to international and departmental guidelines. All patients received ciprofloxacin and itraconazole prophylaxis. Treatment episodes were classified as proven IA, probable or possible IA based on the definitions of the Invasive Fungal Infections Cooperative Group of the European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC-IFIG/MSG). The primary end-point was the use of a broad-spectrum antifungal agent. Results. One hundred and twenty-five patients, representing 145 potentially eligible episodes, were screened. Forty-seven patients with 52 episodes were randomized. Of the 27 episodes in the monitoring arm, the primary end-point was reached in 9 (33.3%). Two cases of IPA were diagnosed as a result of positive GM. One patient was classified as a failure of the presumptive strategy as IMI was diagnosed clinically while undergoing GM monitoring. Of the remaining 24 episodes, CAV was started empirically in 6, despite persistently negative GM results. False-positive GM readings occurred in 9 in the monitoring arm. Of the 25 episodes in the observational arm, CAV was started empirically in 10 for persistent FN. One patient had CT features of IPA. In the intent-to-treat analysis, the presumptive approach saved 11% of patients from empirical antifungals, while in the evaluable-episode analysis, the presumptive approach saved 14%. Twelve-week survival was 85.2% in the monitoring arm and 84% in the observational arm. Conclusions. A presumptive approach to antifungals has the potential to be an alternative to empirical antifungals in the persistently febrile neutropenics. However, one must recognize that the GM assay may not be the ideal diagnostic trigger as clinically significant mold infections (fusariosis, scedosporiosis, zygomycosis) are not diagnosable by this.

0861

IMPACT OF MULTIRESISTANT P. AERUGINOSA BLOODSTREAM INFECTIONS ON OUTCOME OF HAEMATOLOGICAL PATIENTS: RESULTS OF A PROSPECTIVE SURVEILLANCE PROGRAM

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Background. Mortality rate of bloodstream infections (BSI) among neutropenic cancer patients ranges between 5-10%. However, little is known about the real impact on outcome of single pathogens, particularly if multiresistant. *Aims.* Since June 2004, a prospective epidemiological surveillance program is ongoing at our Haematology Unit. At the beginning of 2009, as a consequence of environmental contamination (EHA 2009, abstr. n. 1025), an outbreak of multiresistant P. aeruginosa (MR Pseud), was detected. Herein we determined the impact of MR Pseud BSI on the outcome of haematological patients at our Institution. *Methods.* BSI consecutively occurred at our Institution during a 70 month period (June 2004-January 2010) were evaluated and correlated with type of pathogen, state of underlying disease, neutropenia (<0.5 x $\times 10^9$/L), exposure to previous antibiotic therapy, including prophylaxis with fluoroquinolones, resistance to antibiotics and outcome. Beta-lactam +/- aminoglycoside +/- vancomycin was the regimen adopted for empiric antibiotic therapy in neutropenic patients with fever. P. aeruginosa showing resistance to >1 class of antibiotic with antipseudomonal activity was defined "multiresistant". *Results.* During the observation period, 445 BSI were recorded. Fungi were responsible for BSI in 6 (1.3%) cases (all Candida spp); Gram-positive (G+) bacteria in 150 (33.7%), Gram-negative (G-) bacteria in 256 (57.5%). In 34 (7.6%) cases a polymicrobial BSI was observed. Overall, 67 P. aeruginosa BSI were recorded; 28 out of 67 were MR Pseud. Crude mortality for all BSI was 46/445, 10.3%. Lethality was 3/6 (50%) for fungi, 6/150 (4%) for G+, 29/256 (11.3%) for G-, 8/34 (23.5%) for polymicrobial BSI. Death due to P. aeruginosa BSI was observed in 17/67 cases (25.4%) (MR Pseud: 11/28, 39.3%). Univariate statistical analysis showed that fungal BSI (P=0.016) as well as polymicrobial BSI (P=0.016) and uncontrolled haematological disease (P<0.0001) were predisposing factors for death. Considering single pathogens, only P. aeruginosa was responsible for an increased risk of death (17/67, 25.4% *vs.* 29/378, 7.7%) (P<0.0001) and, in this subset, MR Pseud fully accounted for the increased risk (11/28, 39.3% *vs.* 35/417, 8.4%) (P<0.0001). Neither polymicrobial BSI, nor P. aeruginosa BSI (including MR Pseud) were associated to a diagnosis of acute leukaemia, status of disease, neutropenia or previous exposure to levofloxacin/other antibiotics. In 24 cases of MR Pseud data about empiric first-line antibiotic therapy were available and its relative appropriateness according to antibiogram was evaluated. All but two MR Pseud showed susceptibility to amikacin; in 5 cases aminoglycosides were the only class of active antibiotics. In 6 cases first-line empiric therapy was inadequate; 5 out of 6 patients receiving inadequate treatment died, in comparison with 4/18 receiving at least 1 class of active antibiotics (P=0.017). Summary/*Conclusions.* Together with uncontrolled disease, fungal and polymicrobial etiology, MR P. aeruginosa BSI, wase responsible for an increased risk of death among haematological patients admitted at our Institution. In this setting, beta-lactam+aminoglycosides combination as early empiric antibiotic strategy reduced the mortality when compared to the use of single agent empiric therapy.

0862

THE DETECTION OF GALACTOMANNAN IN BRONCHOALVEOLAR LAVAGE FLUID FOR THE DIAGNOSTICS OF INVASIVE PULMONARY ASPERGILOSIS IN HEMATOONCOLOGICAL PATIENTS

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Background. Invasive pulmonary aspergilosis (IPA) is a severe infectious complication of hematological patients and its early diagnosis can contribute to improve survival of affected patients. *Aims.* To compare the value of galactomannan (GM) detection in bronchoalveolar lavage (BAL) fluid in patients with proven/ probable IPA and patients without IPA. *Methods.* A retrospective analysis of GM detection (Platelia Aspergillus, Bio-Rad, France) in BAL fluid performed in 286 hematology patients between 7/2003 and 12/2009. IPA (EORTC/MSG 2008 criteria) was documented in 17% (50 pts.: proven IPA 7 pts., probable IPA 43 pts.). 28% (79 pts.) with possible IPA were excluded and 55% (157 pts.) without evidence of IPA were considered as negative control. *Results.* 207 samples from 201 pts. were included into analysis. The mean BAL GM index for patients with proven and probable IPA was 1.53 (range 0.07-9.01) and for negative control 0.15 (range 0.07-0.65). For patients with proven IPA mean BAL GM was 2.83 (range 0.12-9.01) *vs.* 1.31 (range 0.07-8.58) for pts. with probable IPA. The subgroup analysis (pts. with proven and probable IPA) shows that the mean value of GM for neutropenic pts. was higher as for non-neutropenic patients (0.86 *vs.* 0.29). 50% pts. with IPA (25/50) had also positive GM in blood sample (cutoff 2x ≥0.5). 70% pts. (35/50) from the group with IPA received mold-active antifungal drug (as prophylaxis or empirical therapy) before

BAL was performed, with median 2 days (range 1 - 60 days). 50% (3/6) of pts. with proven IPA and 45% (13/29) of pts with probable IPA on antifungals at the time of BAL performance had GM index < 0.5 in BAL fluid. The performance of galactomannan in BAL fluid tested at various cutoffs is presented in Table. *Summary/Conclusions.* Our study shows limited sensitivity and high specificity and high positive predictive value of GM detection in BAL fluid in pts. with hematological malignity. Limited sensitivity can be due to high rate of mold-active therapy and prophylaxis at the time of BAL performance.

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Table. Performance of BAL GM tested at various cutoffs.

cutoff BAL GM	sensitivity	specificity	PPV	NPV
≥0.5	54%	99%	90%	87%
≥0.7	46%	100%	100%	85%
≥1.0	38%	100%	100%	83%

0863

VORICONAZOLE PLASMA CONCENTRATIONS IN HEMATOONCOLOGICAL PATIENTS

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Background. Voriconazole (VORI) is a broad spectrum antifungal agent, which exhibits a wide inter- and intrapatient variability of its plasma concentrations.VORI plasma levels monitoring could help us to optimize the individual dosing and improve the efficiency of antifungal treatment. *Aims.* To evaluat VORI plasma concentrations achieved during the VORI treatment in hematooncological patients in our institution. *Methods.* Trough VORI plasma concentrations were measured using a high-performance liquid chromatography assay. Retrospective analysis of laboratory results and documentation from patients treated with VORI from August 2005 to January 2010 was performed.

Table.

PC=plasma concentration

Daily dose	200 mg	400 mg	600 mg	800 mg
samples	81	564	109	31
patients	24	145	29	11
VORI PC <1 μg/ml	75% (n=60)	46% (n=257)	56% (n=61)	41% (n=13)
VORI PC <0.2 μg/ml	13% (n=10)	10% (n=58)	10% (n=11)	9% (n=3)
VORI PC >5 μg/ml	n=4	n=12	n=2	n=4
Mean VORI PC μg/ml	0,94	1,4	1,24	2,3
Median of VORI PC μg/ml	0,66	1,09	0,81	1,08

Results. 836 plasma samples from 148 patients were analyzed. The voriconazole plasma concentration was obtained once in 17% (n=25) patients and twice or more times (2-29) in 83 % (n=123). The monitoring time was 4-420 days after the start of voriconazole treatment. VORI was administered in 38% as prophylaxis, in 10% as an empirical antifungal treatment, in 48% as a preemptive treatment of invasive fungal infection. Only in 4% of cases was voriconazole administered as a treatment of proven invasive fungal infection. The total daily dose of voriconazole was from 200 to 800 mg. Achieved voriconazole plasma concentration varied between 0 and 13.41 μg/mL. 9.8% (n=82) of all obtained samples were below the detection limit of the metod (< 0.2μg/mL). Median plasma concentration achieved after standard daily dose of VORI (400 mg/day) was 1.35 μg/ ml (range: 0.2-7.64) after oral administration and 1.47 μg/ ml (range: 0.2-13.41) after intravenous administration. In 257 samples (45.6 %) after standard daily VORI dose 400 mg the plasma concentration was under 1 μg/mL - below the level associated with a better response to therapy of invasive aspergillosis. The increase of daily dose from 400 to 600 mg lead to elevation of VORI plasma level ≥ 1 μg/mL in 12 cases and in 10 patients the concentration did not changed or decreased. In 12 samples (2%) in 12 patients after the standard dose of VORI (400 mg/ day) plasma level was ≥ 5 μg/mL. However, only in 3 patients toxicity was observed (1 neurotoxicity and 2 hepatotoxicities). *Conclusion.* Measurement of VORI plasma levels during its treatment enables to achieve optimal dosing and an effective concentration, which improves the response to antifungal treatment in hematological patients with high risk of invasive fungal infection. In some cases also could help to identify the side effects and toxicity associated with VORI treatment.

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0864

POSACONAZOLE PLASMA LEVELS MONITORING IN HEMATOLOGICAL PATIENTS

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*Background.*Posaconazole (POSA) is a broad spectrum antifungal used in prophylaxis and treatment of invasive fungal infections in hematological patients.Due to its different oral bioavailability and potential drug interactions POSA exhibits variability in plasma concentrations. *Aims.* To analyse POSA plasma concentrations obtained during the POSA treatment in hematological patients in our institution.*Methods.* The high-performance liquid chromatography assay was used for the identification of POSA plasma concentrations. Retrospective analysis of laboratory results and clinical data from patients treated with POSA from November 2006 to January 2010 was performed. *Results.* 110 plasma samples from 25 patients were analyzed, 1-17 samples per patient. POSA plasma concetrations have been monitored in single patient once (n=10), twice (n=5) or more then three times (n=10) and it was 3-205 (median 31) days after start of POSA therapy. POSA was administered in 55% as prophylaxis, in 32% as a preemptive antifungal theraphy, in 7% as an empirical antifungal treatment and in 6% as a treatment of invasive fungal infection. POSA was administered as an oral suspension with some fat food and the total daily dose varied from 200 to 980 mg, divided in 2-4 portions. In 30% (n=32) of all samples did not achieve the concentration 0.5 μg/mL and thus the treatment could be insufficient.The POSA plasma concentration after daily dose 600 mg (3×200) was 0.2-3.1 μg/mL (median 1.12). After 800 mg daily the plasma concentrations varied between 0 and 3.1 μg/ mL, median was 0.7 and 1.01 after 4×200 mg and 2×400 mg, respectively. There were observed a considerable increase of POSA plasma concentrations in two patients after the change of POSA administration schedule from 2×400 mg to 4×200 mg/ daily.*Conclusion.* POSA plasma concentrations monitoring could be useful guide during the antifungal treatment. It help us to achieve the effective plasma and tissue concentrations and thus could improve the outcome of invasive fungal infection in hematological patients.

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Table.

Daily dose	400 mg	600 mg	800 mg (2x400)	800 mg (4x200)
Samples	2	48	16	35
Patients	2	14	16	35
POSA PC <0.5 μg/ml	100 % (n=2)	23% (n=11)	44% (n=7)	26% (n=9)
Mean POSA PC μg/ml	0.35	1.17	0.78	0.99

PC=plasma concentration

0865

POSACONAZOLE IN PROPHYLAXIS OF FUNGAL INFECTIONS IN NEUTROPENIC PATIENTS A SINGLE CENTRE EXPERIENCE

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Invasive fungal infections (IFI) in patients affected by hematologic malignancies remain an important cause of morbidity and mortality. In attempt to prevent the onset of IFI antimycotic prophylaxis or pre-emptive treatment have been proposed but their role and efficacy are still uncertain. Introduction of Posaconazole as prophylactic agent seems reduce the onset of IFI in patients undergoing stem-cell transplant; it is also recommended in patients with Acute Leukemia undergoing induction chemotherapy. Timing of prophylactic approach is also controver-

sial: there's no evidence, in term of number and evolution of IFI, showing better results in early prophylaxis (in patients with high risk of IFI not yet neutropenic) rather than prophylaxis in febrile persistent neutropenia. We investigated the efficacy, safety and feasibility of Posaconazole as prophylactic agent against IFI in patients with anticipated neutropenia (N<500/mmc), all hospitalized in our Department. During a 18 months period (July 2008 - January 2010) we enrolled 76 adult patients all submitted to intensive chemotherapy or autologous stem cell transplant; the expected severe neutropenia was at least 7 days. Patients with evidence of previous fungal infection were excluded. Patients received posaconazole oral suspension 600 mg from day 1 of chemotherapy until neutrophyls recovery (>500/mmc). Study failure was defined by the following events: start of empiric antifungal therapy for persistent FUO; antifungal therapy for proven or probable IFI; early death; toxicity resulted in too short prophylaxis period (<7-10 d) and/or assumption of <75% fixed dose. We enrolled 39 males and 37 females, median age 53 years (range 19-72), the median prophylaxis period was 20.5 days (range 6-42); 46 patients underwent intensive chemotherapy and 28 pts underwent conditioning regimens for autologous transplant. Diagnosis were: Acute Myeloid Leukemia 31 patients; Acute Lymphoblastic Leukemia 13 patients; Lymphoma 17 patients; Multiple Myeloma 9 patients. The median duration of neutropenia was 20,5 days (range 4-42). 66 of the 76 patients (86%) had at least one episode of fever, in 44 patients the fever duration was less than 3 days. The median duration of fever was 2 days (range 1-16). We observed 18/76 failure (24%), 6 patients (8%) assumed <75% of the fixed dose. IFI proven or probable was diagnosed in 5 patients (6.5%) and possible IFI in 7 patients (9.2%), all but one were acute leukemia patients. 4 patients (5%) died, 3 of them while treated for IFI. Tolerability was very good in 56/76 patients (>90% dose taken) and good in 7/76 patients (>80%). Hepatic or gastrointestinal toxicity grade 2/3 was observed in 13/76 pts (17%). These encouraging results suggest that Posaconazole used in an early phase of intensive chemotherapy can reduce the number and duration of febrile episodes as well as of IFI proven or suspect in a high risk population of hematologic patients.

0866

MALARIA: NEW DIAGNOSTIC APPROACHES

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Background. Migrations have turned malaria into a re-emerging worldwide health problem. *Aims.* To present the demographic, clinical and laboratory features of all cases of malaria diagnosed in our hospital. *Material and Methods.* Study period: January 1998 to December 2009. Diagnostic *Methods.* peripheral blood smear, rapid immunodiagnostic test (Leti Laboratories ®) (for cases diagnosed between 2002 and 2009) and Polimerase Chain Reaction (PCR), since 2008. PCR for malaria has been included in the battery of analytical tests ordered for first-time consultation immigrant patients seen in our Tropical Medicine Unit who have been less than one year in our country. *Results.* the study comprises 55 patients (49 male, 6 female): average age 30 years (range 3-71). Most frequent countries of origin: Senegal (9 cases), Ghana (9), Mali (8), Nigeria (7), Guinea-Bissau (7), Equatorial Guinea (4) and Mauritania (3). Of 25 cases up to 2007, 2 were infested with Plasmodium vivax (385 and 180 days of residence in Spain), and the rest P. falciparum in patients who, coming from their native countries, had arrived to Spain in the 30 days prior to diagnosis. After 2007: 30 cases, including one double parasitization (falciparum+ovale), 2 cases of P. malariae (3 and 4 years of residence), 1 case of P. ovale, and 26 P. falciparum. Among these, 5 were children, 6 women (1 pregnant) and 10 patients who had stayed in Spain between 3 and 8 months. Since the implementation of routine screening, 10 cases have been diagnosticated only by PCR (9 P. falciparum and 1 P. ovale). All others presented clinical features: fever, headache, malaise, abdominal discomfort, while only one presented with serious cerebral malaria. Among hematological abnormalities, the mos frequent were a low platelet count (34/55, 61,8% of total), 34/45 (76% in those diagnosed by blood film observation, none of those diagnosed by PCR) and anemia (25/55, 45,4%; 22/45, 49% diagnosed by blood smear, only 3 diagnosed by PCR). *Conclusions.* Malaria cases diagnosed in our hospital are immigrants. Thrombocytopenia is the most common finding in the complete blood count, not so in asymptomatic patients. We are witnessing a demographic change from 2007 with the appearance of cases in children and women. Interestingly, we have found cases of P. falciparum in patients who have been in our country continously for longer than 10 months. The analytical systematic screening has proved useful in unveiling cases of unsuspected parasitizations.

0867

H1N1V INFECTION IN CHILDREN WITH DREPANOCYTOSIS

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Background. with the new Influenza A H1N1virus it was expected more severe infection in patients with drepanocytosis (sickle cell homozygote hemoglobinopathy). *Methods.* retrospective, descriptive study, of drepanocytic children with H1N1v infection confirmed by Polymerase Chain Reaction observed at a tertiary hospital from October to December 2009. *Aims.* analysis of demographic, vaccinal, hospitalization, clinical, laboratory, radiological features, treatment, complications and outcome of sickle cell patients with H1N1 infection. *Results.* 74 patients are followed at this tertiary hospital, 15 (20%) of whom had H1N1v infection. The majority (11) occurred in November and December. Mean age 7 years (min-1,8; max-15), 10 were admitted. Thirteen patients were not vaccinated with the pandemic vaccine, but 2 had had a first dose less than 3 weeks before infection. Frequent symptoms were: fever, cough and pain. The laboratorial features (73%): haemoglobin (mean-7.9 g/dL, min-4.8), reticulocytes (mean-260000/µl, min-28000), leukocytes (mean-12700/µL, min-11500, max-20300), CRP (mean-0.75 mg/dL, max-14.4). Chest radiography (12): interstitial infiltrate (6), pneumonia (1). Treatment included: oseltamivir (15), antibiotics (4), transfusion (3). Complications were noted: hypoxemia (5), severe anaemia with reticulocytopenia (2), pneumonia (1), moderate to severe pain (3). Mean time of admission was 5 days (min-3, max-21). No deaths occurred. Summary. drepanocytic patients are a risk group for complications of influenza infection and therefore should have the pandemic vaccine. Most cases occurred in November-December, reflecting the importance of early and complete immunization schedule. Nevertheless, although some complications did occur, the general outcome was good.

0868

ASSESSMENT OF HEMATOLOGY PATIENTS WITH CONFIRMED H1N1 POSITIVITY

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Background and *Aims.* Pandemic influenze virus known as H1N1 causes serious complications in immunosuppressive patients. Morbidity and mortality risks are increasing in patients with hematological disorder and have been treated with chemotherapy and stem cell transplantation. In this study, clinical and laboratory findings of hematology patients whose H1N1 positivity has been confirmed while under treatment in our clinic. *Methods.* H1N1 Swine Influenza Suspicious Case Notification Form and Inpatient Follow-up Form have been prepared for 15 patients with a suspicion of H1N1 infection (4 women, 11 men) among inpatients that are being treated at our clinic as of October 2009. Nasopharyngeal swab taken form patients have been forwarded to our virology laboratory. Pandemic influenza A 2009 H1N1 virus was detected by real time RT PCR assay by under the guidance of producer's instructions. *Results.* Nasopharyngeal swab specimens taken from 15 (11 men, 4 women) patients with a suspicion of H1N1 infection. Among 15 patients, H1N1 positivity has been confirmed in 9 patients (7 men, 2 women; age average 49) using real-time PCR. One of 9 patients had been followed due to aplastic anemia, one due to Evans Syndrome, and the remaining 7 due to hematological malignity. Most prominent symptoms are as follows; high fever, cough, vomiting, nausea, diarrhea respectively. 3 patients had concurrent bacterial infection and in one patient fungal infection was present. All patients have been treated with oseltavimir treatment (10 days for patients with pneumonic infiltration, 5 days for the other patients) 8 patients have responded to the treatment and recovered clinically. One patient died due to severe sepsis and pneumonia. *Summary/Conclusion.* Subjective findings like headache and fatigue often seen in influenza infections are seen not to be dominant for these patients. It is remarkable that nausea and diarrhea that are prominent findings for the pandemic H1N1 influenza infections have rarely been detected in these patients. Most of our patients have recovered with intensive support treatment and anti-viral treatment. However one

patient with severe pneumonia died in the clinical course. Associating unaccountable high fever with the currently seen infections and early sampling can be life saving and clinicians should be aware of potential pandemic influenza H1N1 in patients with hematological disorder.

0869

CHARACTERISTICS OF SEPTIC COMPLICATIONS IN PATIENTS, RECEIVING CHEMOTHERAPY, STEM CELL TRANSPLANTATION AND IMMUNOSUPPRESSIVE TREATMENT OF GRAFT-VERSUS-HOST DISEASE

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Background. Patients, receiving intensive chemotherapy of hematologic diseases, hematopoietic stem cell transplantation (HSCT) and immunosuppressive treatment of its main complication, "graft-versus-host"disease (GVHD), are known to bee under extremely high risk of bacterial and fungal infections, which severity varies from neutropenic fever to septic shock. Trends in etiology, risk factors and treatment strategy of severe sepsis and septic shock are well described in literature. Nevertheless in the majority of studies concerning sepsis didn't include hematological and transplanted patients. *Aim.* This study was provided to characterize the etiology of septic complications, factors which determinates mortality from sepsis in patients receiving chemotherapy or HSCT. *Methods.* Forty patients with confirmed diagnosis of sepsis, severe sepsis and septic shock were included in the study. Patients received conventional chemotherapy (72%), HSCT (10%) and immunosuppressive therapy of GVHD after transplantation (18%) for acute leukemia (67,5%), malignant lymphoma (10%), Ewing sarcoma (7,5%), chronic myeloid leukemia (5%), MDS (5%), severe aplastic anemia (2,5%) and SCID (2,5%). 45% of patients were in remission or stable disease at time of sepsis development. The diagnoses of septic complications were provided according to the classical ACCP/SCCM criteria and confirmed with quantitative CRP and semiquantitative procalcitonin evaluation. *Results.* The etiology of sepsis was identified in 77,5% of cases. In 10% of patients sepsis was confirmed by high procalcitonin levels without microbiological verification. In 62,9% of patients sepsis was caused by Gram-negative bacteria and in 25,7% by Gram-positive cocci. Most common bacterial agents were Klebsiella pneumoniae (20%), Pseudomonas aeruginosa (14,3%) and Staphylococcus epidermidis (14,3%). Fungal sepsis was observed in 11,4% of patients. Analysis of antibiotic resistance reveals high rates of multiresistant bacteria (especially Pseudomonas aeruginosa, Klebsiella pneumoniae and Acinetobacter baumanii). Infections caused by these microorganisms were more common for patients admitted to ICU. No cases of septic complications caused by MRSA or VRE were observed. Severe sepsis was observed in 77,5% of patients with the rate of septic shock - 55%. In 70% of cases patients have respiratory failure, in 35% - renal failure, in 35% - liver impairment. Coagulopathy was observed in 42,5% of patients. Overall mortality was 65% in the group, 77,5% in patients with severe sepsis and 68,2% with septic shock. The involvement of liver and kidney in multiorgan failure (MOF) was associated with 100% mortality rate. Another factor, influencing mortality rate was disease status at the time of infection. Patients with stable disease or remission have lover mortality risk, than patients in progression or relapse (39% and 86%, respectively). *Conclusion.* Septic complications in patients with hematological disorders, solid tumors and after HSCT stay an actual problem because of high mortality rates especially in cases of MOF. Risk of mortality is also influenced by the disease status. Most common microorganisms causing sepsis in this high-risk group are multi-drug resistant Gram-negative rods. In the absence of microbiological verification of sepsis the determination of procalcitonin level became valuable diagnostic tool. These factors should be taken into account while planning the treatment of the patient.

0870

HIGH DOSE LIPOSOMAL AMPHOTERICIN B (HD-L-AMB) IN THE PROPHYLAXIS OF INVASIVE FUNGAL DISEASES (IFD) IN ADULT AND ELDERLY ACUTE LEUKEMIA (AL) PATIENTS (PTS)

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Background. IFDs cause morbidity and mortality in AL pts , with an high attributable mortality rate when filamentous fungi are involved. Prophylaxis is an used strategy because the diagnosis of IFD is often difficult to obtain in due time to establish an early treatment .Oral posaconazole is the drug of choice,although mucositis, impaired dietary intake, proton pump inhibitors, influence absorption with pharmacokinetic variability. In these cases the use of a broad spectrum intravenous drug may be preferred. Animal models demonstrate that L-AMB accumulates in the reticulo-endothelial system for several weeks providing effective prophylaxis even at single high dose. *Aim.* We have conducted an exploratory prospective single centre study to test the safety, efficacy and PK profile of HD-L-AMB (15 mg/kg) as IFD prophylaxis in AL pts during intensive induction chemotherapy. Patients and *methods.* From 2004 to 2009, 44 AL pts (AML 40 , ALL 3 , MPAL 1) entered this study. They were 27 adult pts (Group A) - median age 49 years (range 32 -59)- and 17 elderly pts (Group B)- median age 68 years (range 61-78)-; in 3/17 a pulmonary infection was documented at diagnosis and cured before start of chemotherapy. Pts received first HD-L-AMB the day after last dose of chemotherapy . A 2nd dose was given two weeks later in the event of persistent neutropenia in pts without sign of IFD . L-AmB plasma levels were measured by chromatography at d 1st (0,1,4 ,24 h), d 7th and d 14th. To date, PK data are available in 9/26 pts in group A and 8/17 pts in group B. *Results.* The median dosage of administered L-AMB was 900 mg (range 500-1200) in both groups. In Group A, median duration of neutropenia was 22 d (range16 -30) and 3 pts received a 2nd dose. Median L-AMB serum levels at 0h,1h, 4h, 24h, 7th d and14th d were 0, 7.76 , 23.09, 11.8, 0.23 and 0 mg/L, respectively. No IFD was documented at 28 days of follow up. In Group B, median duration of neutropenia was 21 d (range 11-42) and 3 pts received a 2nd dose. Median L-AMB plasma level at 0h, 1h, 4h, 24h, 7th and14th d were 0, 5.4, 13.9, 7.79, 0 and 0 mg/L, respectively. In this group a fatal invasive aspergillosis was documented in the 3 above patients with pulmonary co-morbidity at onset. A serious adverse event CDC grade 3-4 (allergy) requiring drug discontinuation was reported in only one patient of Group A. *Conclusions.* Our preliminary experience seems to show that a single HD-L-AMB is tolerated and safe in adult and elderly AL pts. This antifungal regimen was able to prevent IFDs in pts without co-morbidities involving the respiratory tract. A single HD-L-AMB may be an alternative antifungal prophylactic regimen in AL pts with gastrointestinal problems impairing the absorption of posaconazole. Although no correlation between PK and efficacy can be made with the few available data, the possibility of a less favourable PK profile in elderly pts should be carefully assessed.

0871

PREVALENCE OF SUBTYPES OF HUMAN IMMUNODEFICIENCY VIRUS CIRCULATING AMONG BLOOD DONORS IN PERNAMBUCO, BRAZIL

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Background. AIDS is a disease manifested after infection with Human Immunodeficiency Virus (HIV). It is estimated that about 600 thousand people are living today with HIV in Brazil, which included a total of 40 million worldwide. In Pernambuco were reported about 11 cases between 1980 and 2007 by SINAN (Sistema de Informação de Agravos de Notificação - Information System for Notifiable Diseases). The virus is characterized by a high genetic variability and is classified into two types: HIV-1 and HIV-2. Nine subtypes (A, B, C, D, F, G, H, I, J) are described, in addition to recombinant forms of Circulating Recombinant Forms called. The identification of circulating subtypes is of paramount importance for understanding the epidemic and for future vaccine programs are successful. The natural dynamics of the HIV epidemic in Brazil has led us to evaluate the present subtypes of HIV-1 in blood donations infected with HIV. Blood donors represent a broad cross section of the population of selected groups of high risk and therefore may provide a broader view of circulating strains of HIV-1 in Brazil. *Aims.* The objective of this study is to determine the prevalence of circulating subtypes of HIV-1 in blood donors of the HEMOPE Foundation. *Methods.* We analyzed 40 blood samples from blood donors positive for HIV-1 of the HEMOPE Foundation, being performed DNA extraction of lymphocytes and amplification of the region ENV (gp120 - C2V3 region) of the integrated viral DNA. From this procedure, the glycoprotein 120 gene was amplified by nested-PCR and subjected to automated sequencing. The subtype assignment was confirmed by bootstrapped phylogenetic analysis using SEQBOOT with 1000 replicates,

followed by DNAdist (with Kimura 2-parameter method and transition/transversion ratio of 2.0), Neighbour and Condense programs contained in PHYLIP (PHYlogeny Inference Package). *Results.* Of the 40 samples sequenced, we identified 35 samples (87.50%) of subtype B, 4 samples (10.0%) were subtype C and 1 sample (2.50% [-]) was the recombinant form CRF02_AG. In the rest of Brazil, subtype B is also the most frequent, except in the southern region where the predominant subtype is subtype C. *Conclusions.* The results indicate a greater prevalence of subtype B in the Northeast, especially in Pernambuco, which is consistent with work already published. The preliminary findings of this study demonstrate the need for studies to monitor the epidemiological profile of HIV, especially in regard to screening of circulating viruses and the development of a vaccine program. Also, a clinical follow-up of these donors with defined subtypes can generate information about the specificity of the response of each viral subtype, working with specific treatments.

0872

PNEUMOCYSTIS JIROVECI PNEUMONIA IN AN ONCOLOGY POPULATION, NEW LESSONS FOR AN OLD INFECTION

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Background. Pneumocystis jiroveci is an opportunistic organism and a common cause of pneumonia in patients with impaired cellular immunity. Pneumocystis jiroveci pneumonia (PCP) is the commonest opportunistic infection in AIDS patients and carries a mortality rate of 10-30%. In non-HIV patients with impaired cellular immunity, due to high corticosteroid use, chemotherapy or malignancy, PCP has a mortality rate of 30-60%. With oncology patients living longer, the exact risk and optimum management incorporating investigation, treatment and prophylaxis for PCP is now unclear. *Aim.* To assess the risk factors involved in the development of PCP, to review its diagnosis and the use of PCP prophylaxis in a non-HIV cancer population. *Methods.* A retrospective case note analysis of all patients who were treated for PCP with IV cotrimoxazole between 2006 and 2009 (inclusive) was conducted. Data collected: Malignancy, Risk Factors (corticosteroid in previous 2 months, chemotherapy, immunosuppressants in previous 12 months, previous organ transplantation, previous PCP infection), PCP Prophylaxis, Clinical Presentation (symptoms, onset, SaO2, temperature, lymphocytes count, time to treatment), Diagnosis (definite *vs.* probable) and Outcome. *Results.* 14 patients were included in the study, 9 had haematological malignancies (4 Lymphoma, 3 Multiple Myeloma, and 3 CLL) and 6 had solid malignancies (1 each of NSCLC, SCLC, Cholangiocarcinoma Rectal, and Breast). 2 had early stage cancer, 6 had relapsed, 3 metastatic and 2 were palliative. Risk factors: 12 received corticosteroids in the 2 months prior to infection (daily average 26 mg). 13 received chemotherapy, 7 received other immunosuppressives, and 3 received radiotherapy in the 12 months prior to infection. On presentation, all had signs of symptoms of pneumonia (dry cough, dyspnoea, or lung crepitations), 10 were lymphopenic, 9 were hypoxic (4 severely) and 8 were pyrexic. Diagnosis: 6 patients had definitive PCP diagnosis (5 Bronchoalveolar lavage and 1 expectorated sputum positive for P. jiroveci DNA), 7 had probable (clinical) diagnosis and 1 patient was negative for P. jiroveci DNA in Bronchoalveolar lavage. There were no incidences of failed prophylaxis. Antibiotic treatment for PCP commenced on average 3.3 days after admission. 12 patients recovered from the infection and 2 died. *Conclusion.* Development of PCP was associated with a diverse range of risk factors and a heterogeneous array of malignancies. However, the study highlighted several important points: - Despite the varied chemotherapy received, 85% of patients received corticosteroids in the 2 months prior to infection. - 5 of the 6 Bronchoalveolar lavages tested for PCP DNA were positive, proving it to be a very advantageous investigation. - The mortality rate of PCP amongst patients was better that expected (14% *vs.* 30-60% in the literature). This is a reflection of the prompt initiation of PCP treatment (3.3 days) and emphasises the importance of having a high level of clinical suspicion and a low threshold for treatment. - 4 lymphoma patients (>5% of lymphoma population) were included in the study, highlighting the need to further investigate whether this group is suitable for PCP prophylaxis.

0873

HEMATOPOIETIC STEM CELL TRANSPLANTATION FROM A RELATED DONOR INFECTED WITH SWINE-ORIGIN H1N1 INFLUENZA VIRUS

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Background. Swine-origin H1N1 influenza virus (S-OIV) has caused significant illness worldwide. Complications of influenza infection generally arise in those populations that are most susceptible to infection, such as hematopoietic stem cell transplantation (HSCT) recipients. Among HSCT patients with influenza pneumonia, the 30-day mortality rate can be as high as 28%. Therefore, a guideline for prevention, diagnosis, and treatment of S-OIV is required in HSCT in the pandemic setting, with a concern for transmission of the virus from donor to recipient. *Aims.* We report our experiences of 3 patients whose related donors caught S-OIV during pandemic successfully undergoing hematopoietic stem cell transplantation. Patients and *methods.* From October to December 2009, 26 patients underwent allogeneic hematopoietic stem cell transplantation (13 in the department of medicine and 13 in the department of pediatrics) at Samsung Medical Center. Among them, 10 patients received stem cells from their matched related donors. To detect S-OIV, RT-PCR was performed in various samples including upper respiratory sample obtained by nasal wash, whole blood, and PBSC product. *Results.* Three related donors were infected with S-OIV while recipients (2 patients with severe aplastic anemia and 1 patient with relapsed lymphoma) were treated with conditioning regimens. In two donors, they admitted for PBSC collection a day before transplantation. After admission, they showed fever and cough and tested positive for S-OIV RT-PCR in nasal wash specimen. PBSC collection and transplantation was performed without delay. Donors' blood and PBSC products were also tested for S-OIV by RT-PCR. Although the results were negative in donors' blood, PBSC product showed positive result in one sample. The recipients and donors were given oseltamivir and the recipients did not show any symptoms or signs related to influenza after transplantation. In remaining one donor, he caught S-OIV flu four days before transplantation. He was treated with oseltamivir and he showed progressive leucopenia. G-CSF was given after recovery of leukocyte counts, therefore, transplantation was deferred for five days. In this case, PBSC collection and transplantation were performed after confirmation of negative result on donor's blood and PBSC product by RT-PCR. The recipient was not given oseltamivir because the donor already was treated with oseltamivir for 7 days. The median number of infused $CD34^+$ cells was 5.3×10^6/kg (range 2.0-8.8). Neutrophil and platelet engraftment were occurred at a median of 11 days (range 10-11) and 18 days (range 17-36), respectively. *Conclusion.* The recipients were successfully transplanted from donors infected with S-OIV. Further studies are required to establish a guideline for recipients and donors in hematopoietic stem cell transplantation during influenza pandemic.

0874

ANTIFUNGAL PROPHYLAXIS WITH VORICONAZOLE IN PAEDIATRIC PATIENTS TREATED FOR HEMATOLOGICAL MALIGNANCIES

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Background. Invasive fungal infections (IFI) remain a major cause of death in immunocompromised hosts, such as patients with haematological malignancies receiving chemotherapy, despite the availability of highly effective antifungal agents. The risk of IFI is associated with the degree and duration of neutropenia, the disruption of mucosal barriers and the prolonged use of antibiotics and corticosteroids. Voriconazole is a triazol antifungal agent that exhibits broad antifungal activity against both Aspergillus and Candida (including non-albicans) species, which has been used for the treatment of invasive candidiasis and aspergillosis. Its use as a prophylactic agent is under study, especially in children. *Aims.* the aim of the study was to evaluate the efficacy and safety of voriconazole as antifungal prophylaxis in children suffering from hematological malignancies with chemotherapy induced neu-

tropenia. *Methods.* The files of 69 children (42 Acute Lymphoblastic Leukemia (ALL), 1 mature B ALL, 8 Acute Myelogenous Leukemia (AML), 16 Non-Hodgkin Lymphoma, 2 Hodgkin Disease), were retrospectively reviewed. All patients were treated at a single institution, between January 2006 and December 2009, and received antifungal prophylaxis with voriconazole during chemotherapy induced neutropenia. Age, gender, diagnosis, treatment regimen and laboratory investigations (namely: WBC counts and liver function tests) were recorded. Voriconazole was administered at a dose of 4mg/Kg bid, intravenously or orally. *Results.* In total, 236 patient - cycles were studied. Median duration of neutropenia after chemotherapy during which time voriconazole prophylaxis was administered was 15 days. Treatment success rate defined as absence of proven, probable, possible or suspected IFI was 95.64% (229/236). Proven IFI was documented in two patients with ALL (1 pulmonary aspergillosis, 1 Candida albicans fungemia), one had probable IFI (splenic candidiasis) and in four cases were considered suspected IFIs. No severe adverse reactions or interactions with concurrent medications were observed that could be attributed to voriconazole administration. *Conclusions.* These results suggest that voriconazole, at the described dosage, is an effective and well-tolerated alternative for antifungal prophylaxis in children with hematological malignancies during chemotherapy induced neutropenia. Randomized, double-blind, case-control studies are needed to confirm the efficacy of this approach in comparison to standard treatments.

0875

THE USE OF CELL POPULATION DATA FOR THE DIAGNOSIS OF NEONATAL SEPSIS

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Background. Larger, immature neutrophils pour in the bloodstream during neonatal sepsis. Current technology allows to assess the neutrophil volume distribution, an index that has previously been used to detect acute bacterial infections in the adult. We have studied automated neutrophil volume and its distribution as a screening tool for late onset sepsis in very low birth weight neonates. Patients and *Methods.* Consecutive very low birth weight symptomatic neonates were screened for sepsis using complete blood count (CBC), absolute neutrophil count (ANC), immature /total (I/T) ratio, C- reactive protein (CRP). Neutrophil volume (NeV) and neutrophil volume distribution width (NDW) were determined both in infants with suspected sepsis and in a group of controls matched for gender, birth weight and gestational age who were not symptomatic for sepsis. Blood culture was used as the gold standard for infection. Receiver operator curves, area under the curve, sensitivity, specificity, positive and negative predictive values were calculated for each test. *Results.* We enrolled 120 neonates with suspected sepsis and 60 asymptomatic infants. NeV proved to be a useful diagnostic test for sepsis on a single determination (sensitivity= 95%; specificity=88%; cut off =148 arbitrary units) performing better than CRP (sensitivity= 65%; specificity=96%; cut off =0.9 mg/dL), white blood cells count, ANC and I/T ratio. NDW was of poor value (sensitivity=80% specificity=52% cut-off=27.5) . When CRP and NeV were considered together, sensitivity was unchanged while specificity rose to 97%. NeV was positive in 1 out 60 asymptomatic infants. *Conclusion.* NeV is a reliable and inexpensive adjunct to the current screening tests for neonatal late-onset sepsis.

0876

ASPERGILLUS GALACTOMANNAN (GM) ANTIGEN IN THE BRONCHOALVEOLAR LAVAGE (BAL) FLUID FOR THE DIAGNOSIS OF INVASIVE PULMONARY ASPERGILLOSIS (IPA) IN HEMATOLOGICAL PATIENTS

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Background. IPA is a diagnostic challenge in immunosupressed patients. Recently developed BAL GM assay shows promising *Results.* A positive BAL GM has also been proposed as a criterion of IPA, although it has not been fully validated. *Aims.* Our purpose was to investigate the role of GM in BAL fluid as a tool for diagnosis of IPA in hematological patients. *Methods.* We prospectively collected clinical and laboratory data in patients who had BAL GM performed in Hematology, Oncology and Transfusion Medicine Center of Vilnius University Hospital between 2006 and 2009. A BAL GM cut-off value of ≥ 0.5 was considered positive. Patients were categorized as having proven, probable, or possible IPA according to the revised EORTC/MSG definitions. The sensitivity, specificity, negative and positive predictive values of positive BAL GM were calculated with respect to proven or probable IPA. *Results.* 84 patients had BAL GM performed. 48 (57%) were male and 36 (43%) were female. Median age was 48 years (range 18-84). 9 (10.7%) patients had chronic myelogenous leukemia, 39 (46.4%) had acute leukemia, 9 (10.7%) had chronic lymphocytic leukemia, 13 (15.5%) had lymphoma, and 14 (16.7%) had other hematological diseases. In 44 (52%) cases neutrophil count was $<0.5x10^9$ L. Autologous or allogeneic stem cell transplantation had been performed in 8 and 24 patients, respectively. 2 (2.4%) patients had proven, 41 (48.8%) had probable, and 41 (48.8%) had possible IPA. BAL GM and serum GM were simultaneously obtained in 47 (56%) cases, and both tests were positive in only 4. Chest CT scan was performed in 63 (75%) patients at the time of suspected IPA and was positive in 53 cases. The sensitivity, specificity, negative and positive predictive values of positive BAL GM were 100%, 70.7%, 100% and 77.8%, respectively. *Conclusions*. BAL GM has a high negative but a moderate positive predictive value. Serum GM is only rarely positive in IPA.

Lymphoma - Clinical 3 (including Hodgkin lymphoma)

0877

IMPACT OF FEBRILE NEUTROPENIA ON RCHOP CHEMOTHERAPY DELIVERY AND HOSPITALIZATIONS: RESULTS FROM AN INTERNATIONAL OBSERVATIONAL STUDY IN PATIENTS WITH NON-HODGKIN LYMPHOMA (IMPACT NHL)

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Background. Data indicate that reductions in CHOP (cyclophosphamide, doxorubicin, vincristine, prednisolone) chemotherapy dose intensity below 90% may decrease survival in patients with aggressive non-Hodgkin lymphoma (NHL).[1] Due to its myelotoxicity, the delivery of CHOP chemotherapy is frequently impeded by severe neutropenia and/or febrile neutropenia (FN), particularly if a period of hospitalization is required. *Aims.* In this post-hoc analysis from the IMPACT NHL study, we aimed to describe the impact of FN on chemotherapy delivery. *Methods.* IMPACT NHL included patients (≥18 years) with NHL receiving either CHOP-14 or CHOP-21 chemotherapy±rituximab (R). Centres in Europe (123 sites) and Australia (5 sites) each included the five consecutive, eligible patients that had most recently completed their (R)CHOP chemotherapy, and recruited 10-15 further patients prospectively. This analysis focuses on chemotherapy delivery parameters and unplanned hospitalizations for patients with diffuse large B cell lymphoma (DLBCL) receiving R-CHOP chemotherapy depending on whether or not they experienced FN. *Results.* A total of 1111 patients treated between 2005 and 2008 were included in this analysis (R-CHOP-21, N=702; R-CHOP-14, N=409). Respectively, 36% and 84% of the R-CHOP-21 and R-CHOP-14 groups received G-CSF primary prophylaxis; 133 (19%) and 81 (20%) patients respectively experienced FN. In the R-CHOP-21 group, 47% of patients who experienced FN did so in cycle 1, as did 30% of those who experienced FN with R-CHOP-14 chemotherapy. Patients who experienced FN tended to be older than those who did not, and to have higher risk disease according to the International Prognostic Index (IPI) (Table). More patients with FN had bone marrow involvement and a history of comorbidities than those without FN (*data not shown*). Unplanned hospitalizations were substantially more frequent in patients who experienced FN than in patients with no FN. Chemotherapy dose delays were more frequent in patients who experienced FN than in those who did not (Table), as were dose reductions in the R-CHOP-21 group. Overall, fewer patients with FN received optimal (≥90%) relative dose intensity (RDI).

Table.

	R-CHOP-21		R-CHOP-14	
	FN (N=133)	No FN (N=569)	FN (N=81)	No FN (N=328)
Patient characteristics, n (%)				
Age ≥65 years	85 (64%)	275 (48%)	39 (48%)	129 (39%)
IPI intermediate-high	86 (65%)	285 (50%)	62 (77%)	184 (56%)
Outcomes in any cycle, n (%) [95% CI]				
Unplanned hospitalization	105 (79%) [71%, 85%]	100 (18%) [15%, 21%]	63 (78%) [68%, 85%]	68 (21%) [17%, 25%]
Dose reduction ≥10%†	40 (30%) [23%, 38%]	102 (18%) [15%, 21%]	14 (17%) [11%, 27%]	41 (13%) [9%, 17%]
Dose delay > 3 days*	73/128 (57%) [46%, 63%]	226/563 (40%) [36%, 44%]	61/80 (76%) [65%, 83%]	148/325 (46%) [40%, 51%]
RDI ≥90%†	83 (62%) [54%, 70%]	434 (76%) [73%, 80%]	30 (37%) [27%, 48%]	234 (71%) [66%, 76%]

*denominator is patients who received ≥2 cycles
†Excluding vincristine.

Conclusions. In this study of everyday clinical practice, fewer DLBCL patients received R-CHOP chemotherapy at the planned time and dose if they experienced FN. Unplanned hospitalization was also more frequent for patients with FN - with possible implications for chemotherapy delivery as well as costs. FN frequently occurred in the first cycle. Physicians should consider G-CSF primary prophylaxis for patients scheduled to receive R-CHOP-21 who are at overall FN risk of 20% or higher, and for patients prescribed R-CHOP-14.[2]

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0878

THE NEWLY DEVELOPED PVAG-REGIMEN IS ACTIVE AND FEASIBLE IN ELDERLY PATIENTS WITH INTERMEDIATE OR ADVANCED STAGE HODGKIN LYMPHOMA: RESULTS OF A PHASE II STUDY OF THE GERMAN HODGKIN STUDY GROUP (GHSG)

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Background and *Aims.* About 20% of all patients diagnosed with Hodgkin lymphoma (HL) are older than 60 years. These patients have a rather poor prognosis, particularly when presenting in intermediate or advanced stages. Besides a biologically more aggressive disease, the main reason is a drastically increased toxicity of chemo- and radiotherapy resulting in a higher treatment-related mortality and insufficient dosing of the applied treatment. In the GHSG-HD9 trial, elderly patients did not benefit from the BEACOPP regimen in terms of overall survival due to a high toxicity related death rate. In order to improve tolerability, the PVAG regimen (prednisone, vinblastine, doxorubicin, and gemcitabine) was developed. This is a modification of the ABVD regimen in which bleomycin and dacarbazine were replaced by prednisone and gemcitabine. Here we report for the first time on the final analysis of this multi-center phase II study for elderly HL patients. *Methods.* 61 patients were recruited between 2004 and 2007. 3 patients were excluded due to histology review not confirming HL, resulting in 58 patients with HL in intermediate or advanced stages aged between 60 and 75 years. Treatment consisted of 6 cycles PVAG in patients achieving a complete remission (CR) after 4 cycles or 8 cycles PVAG in case of partial remission (PR) after 4 cycles. Patients who did not achieve CR after the end of chemotherapy received additional radiotherapy. Primary endpoints were administration of adequate dose without excessive delays, and response rate 3 months after end of treatment. Secondary endpoints included WHO grade III/IV toxicities, and occurrence of early progression. *Results.* 58 patients with a median age of 68 years were evaluated, of which 59% were male and 93% had advanced stage disease. The relative dose intensity (relative dose divided by relative chemotherapy duration) was at least 80% in 44 patients (76%). Regarding the single cycles, of which 85% started without major delay (max. 1 day), the mean relative dose of all agents was slightly decreasing over time but always exceeded 90%. WHO grade III/IV toxicities were documented in 43 patients (75%). Only 3 patients terminated CT because of excessive toxicity. 10 Patients (17%) received consolidating radiotherapy. In total, 44 patients responded with CR/CRu (76%), 2 with PR (3%), 2 with no change (3%) and 4 with progressive disease (7%). In the remaining 6 patients, treatment outcome is currently being re-evaluated and final results will be presented. With a median observation time of 31 months, 15 progressions or relapses and 16 deaths have been observed, of which only 1 was due to PVAG related toxicity but 8 were due to HL. The 2-year-estimates for overall survival and for progression-free survival were 82% (95%-CI 67% to 90%) and 64% (95%-CI 49% to 76%), respectively. *Conclusion.* PVAG is safe and feasible in Hodgkin patients older than 60. The PFS indicates activity of this regimen in this poor prognosis patient cohort. However, a controlled randomized trial to determine the best treatment in this patient population is warranted.

0879

TANDEM AUTOLOGOUS/REDUCED-INTENSITY ALLOGRAFT FOR RELAPSED/REFRACTORY HODGKIN'S LYMPHOMA: EARLY ALLOTRANSPLANT AFTER INTENSIVE CYTOREDUCTION MAY MAXIMIZE GRAFT VS LYMPHOMA EFFECT?

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Background. Hodgkin's Lymphoma (HL) patients primary chemorefractory and with disease at the time of autolgous transplantation (ASCT), have high probability of progression after ASCT. Reduced-intensity conditioning allotransplant (RICT) aims to exploit graft *vs.* lymphoma (GvLy) effects while reducing conditioning-related toxicity. RICT is considered as the last therapeutic option and is usually offered to HL patients failing ASCT and in this contest GvLy responses might be insufficient. *Aims.* we pionered that offering RICT as an earlier option after intensive cytoreduction (ASCT) may allow GvLy reaction to be better exploited (Carella *et al*. JCO 2000; 18:3918). Patients and *Methods.* 35 HL patients (21M/14F), 75% primary chemorefractory and 26% relapsed after first line chemotherapy, underwent RICT preceeded by autografting (ASCT), after one (71%) or multiple salvage chemotherapy lines (29%). Patients progressing after ASCT were excluded from the analysis. Median age at diagnosis was 27 (range 15-47). Twentyeight patients (80%) had disease before tandem (CR=7; PR=14; SD=4; PRO=10). High-dose therapy consisted of Melphalan 200mg/mq (n=9) and BEAM (n=26). The time interval between ASCT and RIC was 2.5 months (range 1.5-5.5). RIC consisted of fludarabine-cyclophosphamide (n=20) or fludarabine-melphalan (n=15). Thirty patients were allotrasplanted from an HLA identical donor, four from a MUD and one patient from a family-mismatched donor. *Results.* the median time to neutrophils and platelets recovery was 14 days and 15 days, respectively. Chimerism studies indicated 100% donor-derived engraftment. Eleven patients (31%) developed aGVHD (grade I-IV) and 13 cGVHD (2 limited and 11 extensive). No serious complication occurred after both transplantation procedures. ASCT induced a disease response (CR+PR) in 18/35 patients (51%). After tandem ASCT-RICT, 69% of the patients (CR=22;PR=2) were responsive, 31% (N=11) had persistent disease (PRO=10;SD=1). With a median follow-up of 48 months (range 6-127), 62% of patients (n=22) were alive and of these 82% in CR (n=18) and 18% (n=4) had persistent disesase. Overall, 37% of the patients (n=13) died: 23% (n=8) of progressive disease and 14% in CR (n=5) of toxic events. No patients died before one hundred days. Three patients died before one year and two after three years. Non relapse mortality (NRM) was 8.6% (n=5). Median OS and PFS were 60.5 months and 11.5 months respectively and 3-years OS and PFS was 76.5% and 41.6% respectively. In univariate analysis, disease status before tandem influenced PFS a statistically significant manner (3-years PFS 66.7% (chemosensitive) *vs.* 21.4% (chemoreferactory) (P<0.0002). GVHD did not influence the outcome. Median survival of patients progressing and relapsing after tandem was 36 months. *Conclusions.* with the limits of a retrospective study, these encouraging results suggest that GvLy may have a role on residual disease after ASCT. We can speculate that RICT determines a disease chronicization. A prospective study based on genetic randomization (ASCT *vs.* ASCT followed by RICT) would help to answer these important issues.

0880

RECURRENT HODGKIN LYMPHOMA - IS IT ALWAYS A RELAPSE OF THE ORIGINAL CLONE?

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Background. Though the fast majority of Hodgkin lymphomas (HL) can be cured by multimodal therapy, a small number of patients will experience a relapse. Early relapses seem to be associated with a worse prognosis than late recurrences. Relapses are usually treated with intensive chemotherapy regimens. *Aims.* We investigated if the first and all further manifestations of HL in a patient are morphologically, virologically and clonally related to each other, i.e. if the recurrence is really a relapse of the first tumor or rather an independently arising second neoplasia. *Methods.* We identified a collective of 13 patients with, sometimes multiple (6 patients), recurrences of HL from our archives (Figure 1). Specimens were cut onto adhesive coated slides. Hodgkin and Reed-Sternberg cells were microdissected after immunohistochemical staining for CD30 using the laser-capture technique (PALM microbeam system from Zeiss). Immunoglobulin heavy chain (IgH) fragment lengths were analyzed after DNA pre-amplification and polymerase chain reaction, applying consensus FR3 and J primers by an ABI 310 Genetic Analyzer. A total number of 31 formalin-fixed, paraffin-embedded tissue samples [primary tumors and (multiple) recurrences] were analyzable for clonality. *Results.* Relapses were classified as early, if the recurrence occurred within 12 months after primary diagnosis (two events in two patients) or after a first (three events in three patients) or second relapse (one event), or as late recurrences, if occurring later than one year after a previous diagnosis (15 events in eleven patients) (Figure 1). All two early relapses after first HL diagnosis (cases 2 and 5) were clonally related to the initial tumor, while three of four early relapses after a first or second relapse (cases 10, 11 and 13) were not. Two late relapses (cases 4 and 12) were clonally unrelated to the initial HL, which was accompanied by a switch of phenotype and EBV-association in one case. One patient (case 10) had two simultaneous clonally unrelated early second relapses, at different anatomical sites and with divergent EBV-association, after first recurrence. Another two cases (cases 3 and 9), initially presenting as HL and relapsing as diffuse large B-cell lymphoma or vice versa, showed clonal relationships between both entities. *Summary*. Not all cases of relapsing HL are clonally related to the initial and/or previous neoplasm. Recurrent HL, especially after a relapse has taken place, or those accompanied by phenotypic and/or EBV-association switch, may represent clonally unrelated second neoplasms. Assessment of the clonal relationship on formalin-fixed paraffin-embedded HL specimens is technically feasible and might play a role in clinical decision making.

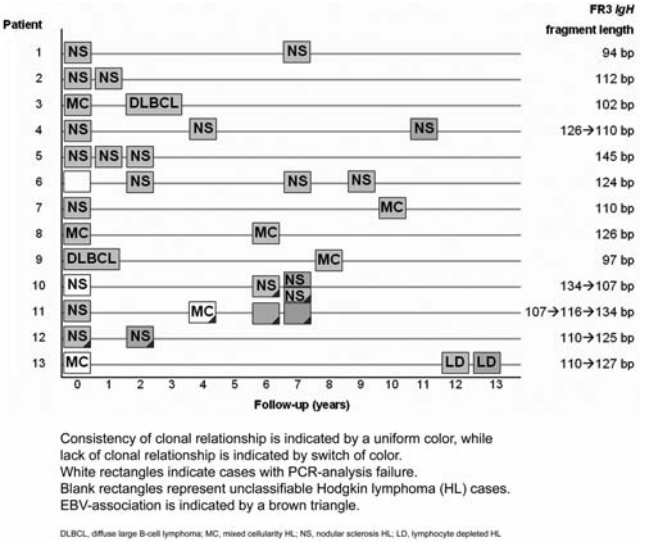

Figure 1. Summary of the analyzed cases.

0881

ABERRATIONS OF THE 9P24 REGION HARBORING JAK2, PDL1 AND PDL2 GENES ARE RECURRENT IN CLASSICAL HODGKIN LYMPHOMA

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Background. Classical Hodgkin lymphoma (cHL) is a clinically well characterized tumor. The biology of this lymphoma, however, is poorly investigated due to the rarity of the neoplastic Hodgkin and Reed-Sternberg (H/RS) cells in the tumor mass, their genetic instability and low proliferation abilities. Interphase FISH studies showed that H/RS cells are characterized by frequent gain/amplification of the 2p16/REL and 9p24/JAK2 loci. The important role of 9p24 aberrations in the pathogenesis of cHL was recently highlighted by our finding of the novel SEC31A-JAK2 fusion as the first recurrent rearrangement in cHL (Van Roosbroeck *et al.,* Blood, 2009, 114: 140). This fusion, associated with the t(4;9)(q21;p24), was identified in two cHL cases and we showed that SEC31A-JAK2 is a constitutively activated tyrosine kinase that has oncogenic potential *in vitro* and *in vivo*. Aim. The objective of this study was to determine the prevalence of 9p24/JAK2 aberrations

in cHL. *Methods.* Interphase FISH analysis with a dual color JAK2 break apart (BA) assay combined with an aqua-labeled centromeric probe for chromosome 8 (for ploidy control) was performed on 137 unselected cHL cases. Several additional 9p24 BACs/fosmids were applied in cases with an aberrant JAK2 BA signaling pattern. Only huge cells (2-10/case), likely representing H/RS cells, were evaluated. Results. Interphase FISH analysis detected aberrant hybridization patterns of the JAK2 BA assay in 72% of analyzed cHL cases. These patterns suggested (i) structural rearrangements (5 cases) and (ii) copy number changes of the JAK2/9p24 region (93 cases). Further FISH analyses of the former five tumors identified one case with a 9p24 breakpoint in the 5' region of JAK2. This aberration possibly represents a novel JAK2 fusion because interphase FISH with 3' JAK2 probes combined with 5' probes of all 6 known JAK2 fusion partners did not show colocalized signals. The remaining four cases analyzed with additional 9p24 probes displayed breakpoints in the region harboring the PDL1 and PDL2 genes mapped 34 kb centromeric to JAK2. In addition, copy number changes of the 9p24/JAK2 region were very frequent in cHL (68%). Besides gain of 1-5 copies (38%) and (clustered) amplification (28%) of JAK2/9p24, we found three cHL cases with a predominant amplification of PDL1/2. Whether these genes, coding for regulators of T cell activation, are targets of the 9p24 rearrangements in cHL is currently investigated. Of interest, previous studies showed that expression of both PDLs is frequently upregulated in cHL cell lines as well as in primary H/RS cells. It has been postulated that tumors use the programmed death 1 (PD1)/PDL pathway to reduce tumor immunity and increase tumor survival. Conclusions. We confirmed that 9p24 aberrations are recurrent in cHL. In rare cHL cases, JAK2 is targeted by rearrangements leading to the generation of oncogenic fusion proteins. In the vast majority of cHL cases, however, the 9p24 region is affected by copy number changes. Given a selective gain of the neighboring PDL genes in a few cases, further studies are needed to define the true target(s) of the 9p24 amplifications in cHL.

0882

AN ANALYSIS ON THE ROLE OF FDG PET IN HODGKIN LYMPHOMA STAGING

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Background. FDG-PET is necessary for the early evaluation (EE) of treatment response in Hodgkin lymphoma (HL) patients, while its use is recommended but not mandatory for disease staging. *Aim.* To evaluate the role of FDG-PET in comparison with TC scan in HL staging. *Methods.* One hundred six new consecutive HL patients seen between 2007 and 2009 at the Hematology Units of the "Sapienza"University of Rome and of "Polo Pontino. We studied 61 males and 45 females; median age was 40 year (range 19-70). The patients were staged with both conventional techniques (CT [TC scan, echography and bone marrow biopsy]) and whole body FDG-PET. The results of CT and FDG-PET staging were compared. *Results.* CT and FDG-PET results were concordant in 96/106 patients (90.7%) while in 10/106 (9.3%) were discordant; of these, 8 (7.4%) were up-staged by FDG-PET: 2 cases became stage III because of a sub-diaphragmatic nodal and spleen involvement not identified by CT, whereas 6 cases advanced to stage IV because of bone (5 cases) and hepatic lesions (1 cases) detected at the FDG-PET (TC scan and Bone Marrow Biopsy = negative). In these 8 cases changes were as follows: from stage II to IV: 3 cases; from stage III to IV: 3 cases; and from stage II to III: 2 cases. In two cases (1.9%) FDG-PET downstaged from III to II. Because of FDG-PET staging results treatment plan was changed in 5 patients. As concern the EE after 2 cycles of ABVD, all the 8 cases upstaged by FDG-PET analysis converted to a FDG-PET negativity. In particular, comparing the groups of patients with FDG-PET and CT stage IV HL, we observed that all the 6 (100%) and 4 of the 12 (33%) patients with a FDG-PET and CT defined stage IV, respectively, converted to a FDG-PET negative rate after 2 ABVD cycles (P=0.01; Fisher's Exact Test). Thus, the 8 persistently PET-positive patients received an intensification treatment with high dose chemotherapy and Autologous Stem Cell Trasplantation. After a median follow-up of 16 months (3-38) all patient up-staged on the basis of PET results are in persistent Complete Remission. *Summary/Conclusions.* Our data confirm a good concordance between conventional methods and FDG-PET. This latter analysis, seems to possess a higher accuracy in detecting bone lesions, as further suggested by present findings showing the early conversion to negativity in all the 8 FDG-PET up-staged patients. In addition, the worst rate of conversion to FDG-PET negativity observed in the "conventional"stage IV group suggests that, among patients with disseminated HL disease, those with a FDG-PET-defined stage IV may have a more favourable clinical outcome. Thus ad hoc future studies are mandatory to define the more appropriate treatment for FDG-PET up-staged patients, to avoid a putative risk of over-treatment of this patient category.

0883

POORER OUTCOME OF LYMPHOCYTE-DEPLETED HODGKIN LYMPHOMA: A COMPREHENSIVE ANALYSIS FROM THE GERMAN HODGKIN STUDY GROUP

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Background. Lymphocyte-depleted classical Hodgkin lymphoma (LDcHL) is a rare entity accounting for approximately 1% of classical Hodgkin lymphoma (cHL) patients, which made analyses difficult in the past. *Aims.* To investigate the clinical characteristics and treatment outcome of patients with LDcHL compared with other histological subtypes of cHL. Patients and *Methods.* From a total of 10,020 patients with biopsy-proven HL treated within the trials HD4 to HD15 of the German Hodgkin Study Group, 84 patients with LDHL (<1%) were identified. Only patients who underwent a central expert pathology review were included. *Results.* Compared with all other histological subtypes, e.g. nodular sclerosis (NS) or mixed cellularity (MC), patients with LDcHL presented more often with advanced stage of disease (74% *vs.* 42%) and B-Symptoms (76% *vs.* 40%). Risk factors including large mediastinal bulk, elevated erythrocyte sedimentation rate, extranodal disease, involvement of 3 or more lymph node areas, and organ involvement of bone marrow or liver were more frequently in LDcHL patients. The median follow-up was 67 months. Complete remission (CR) or CRu (unconfirmed) was achieved in 82% of LDcHL patients compared to 93% of other cHL patients. More LDcHL patients had progressive disease. Progression-free survival (PFS) and overall survival rates were significantly lower in LDcHL patients [PFS at 5 years: 71% (LD) v. 85% (NS) or 84% (MC); P<0.0001)]. However, when analysing the subgroup of patients that underwent modern treatments with intensified or dose dense regimen such as BEACOPP-escalated or BEACOPP-14, LDcHL patients (n=39) had no longer an inferior outcome compared to non-LDcHL patients (n=3564) (P=0.61). *Conclusions.* LDcHL has a different pattern of clinical presentation with more clinical risk factors at presentation than other types of HL. Thus, this entity is associated with a significantly poorer prognosis. LDcHL patients should be treated with modern dose-intense treatment strategies.

0884

A SYSTEMATIC REVIEW COMPARING BEACOPPESCALATED AND ABVD-LIKE CHEMOTHERAPY FOR HODGKIN LYMPHOMA PATIENTS

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Introduction. The main challenge in treating Hodgkin lymphoma (HL) is to find the optimal treatment with the best efficacy and least toxicity. So far there are two different international standards for the treatment of intermediate and advanced stage HL: chemotherapy with ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine) regime and chemotherapy with $BEACOPP_{escalated}$ (bleomycin, etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine, and prednisone) regime. This review aims to clarify advantages and disadvantages of both treatments. *Methods.* MEDLINE, CENTRAL and EMBASE were systematically searched for randomised controlled trials from 1985 to 2008. Trials comparing treatment with at least 2 cycles $BEACOPP_{escalated}$ *vs.* chemotherapy with at least 4 cycles of ABVD for patients in early-unfavorable or advanced stages HL were included. Trials without any published results (e.g. trial 20012 from the EORTC) were excluded from the meta-analysis. Trial selection, quality assessment and data extraction were done independently by two review authors. Time-to-event outcomes were analyzed

with hazard ratios (HR) and 95% confidence interval (CI) in a fixed effects model. *Results.* A total of 683 references were screened. Four eligible trials with 1970 patients were identified and included in the main analysis: the HD9 and HD14 trials from Germany, the HD2000 and GSM-HD trials from Italy. Two trials report toxicity during treatment: the $BEACOPP_{escalated}$ regime causes statistically significant more haematological toxicities WHO grad 3-4 than ABVD-like regimen and statistically significant more infections, alopecia, mucositis and pain. No differences between $BEACOPP_{escalated}$ and ABVD-like regimen were found for constipation, nausea and neurologic toxicity. Progression free survival (PFS) was statistically significantly longer for $BEACOPP_{escalated}$: HR was 0.52 (95% CI [0.43, 0.64], I2=0%). In the main analysis of overall survival (OS), $BEACOPP_{escalated}$ was statistically significantly better than ABVD: HR was 0.71 (95% CI [0.50, 0.99], I2=0%). However, this result is not robust. In the OS-analysis restricted to the 3 advanced stage trials (HD9, HD2000 and GSM-HD), the HR was 0.74 (95% CI [0.52, 1.06], I2=0%). Due to the high weight of the HD9 trial (57%), the overall OS-results are strongly influenced by this trial. In a sensitivity OS analysis without the HD9 trial the HR was 0.83 (95% CI [0.50, 1.38]). Only one trial (HD9) had a follow-up of more than 5 years; the median observation time of the other trials was approx. 3 years. *Conclusions.* This meta-analysis confirmed the higher haematological toxicity of the $BEACOPP_{escalated}$ regime in comparison to ABVD-like regimen. It showed a better PFS and suggests a benefit in OS for $BEACOPP_{escalated}$ in comparison to ABVD-like regimen. However, 3 trials had a short follow-up. Longer follow-up and the inclusion of the ongoing EORTC 20012 trial should allow a more definitive answer concerning OS.

0885

ESHAP VS IGEV AS SALVAGE AND MOBILIZING REGIMENS IN RELAPSED OR REFRACTORY HODGKIN LYMPHOMA (HL)

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Background. The standard treatment approach for patients with relapsed or refractory HL is salvage chemotherapy and subsequent high dose therapy and autologous stem cell transplantation (HDT/ASCT). Salvage chemotherapy aims to disease debulking, testing of chemosensitivity, and mobilization of peripheral blood stem cells. Platinum-based regimens (DHAP, ESHAP, ICE) are frequently used for this purpose. However, the optimal salvage chemotherapy regimen is still not known. Recently the combination of gemcitabine, ifosphamide, vinorelbine and solumedrol (IGEV) has been shown to be an effective salvage and mobilizing regimen in HL. *Aims.* To compare the efficacy of ESHAP (etoposide, methylprednisolone, high dose cytarabine and cis-platinum) *vs.* IGEV chemotherapy as 2nd line treatment for relapsed or refractory HL patients eligible for HDT/ASCT. *Methods.* Between 2001 and 2006 most patients scheduled for ASCT received ESHAP as first salvage (n=37), while IGEV was introduced as first salvage during the last 3 years (n=33). We retrospectively compared these two regimens regarding successful mobilization, disease control (overall response rate) and a combined endpoint, including both successful mobilization and disease control prior to ASCT. *Results.* Patients' characteristics did not differ between ESHAP and IGEV groups, except of bulky disease at relapse/progression, which was more frequent in the latter (P=0.008). IGEV was more effective as a mobilizing regimen: peak circulating $CD34^+$ cell counts were higher (median 198.6 *vs.* 75.2, P<0.001), the number of total CD34+ collected cells was higher (median 11.6×10^6/kg *vs.* 4.32×10^6/kg, P<0.001), while all patients were successfully mobilized with IGEV *vs.* 90% in the ESHAP group. In addition, time to neutrophil engraftment following ASCT was faster with IGEV (median 9 *vs.* 10 days, P=0.002). The median time to apheresis was also shorter with IGEV (12 *vs.* 16 days, P<0.001). Moreover, IGEV was administered as an outpatient regimen. Response (complete and partial remission) rate was similar with both regimens (50% *vs.* 51% with ESHAP *vs.* IGEV). The combined endpoint of successful mobilization and disease control was achieved in a similar percentage of patients with both regimens (60% *vs.* 56%). *Conclusions.* ESHAP and IGEV have comparable efficacy regarding disease control in relapsed/refractory HL patients as salvage chemotherapies. However, IGEV is a more effective and convenient mobilizing regimen compared to ESHAP.

0886

POSITIVE PRETRANSPLANTATION POSITRON EMISSION TOMOGRAPHY INDICATES POOR PROGNOSIS IN RELAPSED HODGKIN LYMPHOMA PATIENTS

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Background. Autologous stem cell transplantation (ASCT) is the standard of treament in relapsed Hodgkin lymphoma (HL) patients however, it cures only half of them. Several risk factors have been studied to determine the risk of relapse after ASCT. *Aims.* This retrospective study evaluated the secondary clinical risk score at relapse, the prognostic significance of pretransplantation positron emission tomography (PET) and achievement of complete remission (CR) assessed by CT after salvage chemotherapy before ASCT in 76 relapsed/refractory HL patients. *Methods.* Progression-free survival (PFS) and overall survival (OS) of patients was calculated using Kaplan-Meier method. Differences of survival between subgroups were analyzed with log-rank test. *Results.* Median follow-up of patients after ASCT was 23 months. Overall 11 (55%) of 20 PET positive patients and 14 (25%) of 56 PET negative patients relapsed after ASCT. The 2-year PFS in PET negative *vs.* PET positive patients was 72.7±6.3% *vs.* 36.1±11.6%, respectively (P=0.01). The 2-year OS in PET negative *vs.* PET positive patients was 90.3±4.1% *vs.* 61.4±11.6%, respectively (P=0.009). In univariate analyses, the secondary clinical risk score at HL relapse, primary refractory disease or achievement of CR evaluated according to CT scan was not statistically significantly associated with PFS and OS. In multivariate analysis PET was statistically significant for PFS only in combination with other variables (clinical risk score and CR assessed by CT) (P=0.017). Multivariate analysis of these variables, however, was not significant for OS (P=0.08). Conclusion. Positive pretransplantation PET identified a high-risk subgroup with worse overall survival when comapared to PET negative patients. This high-risk patients may benefit from further treatment after ASCT.

0887

BASELINE SERUM C-REACTIVE PROTEIN LEVELS (CRP) IN HODGKIN LYMPHOMA (HL) AND ALTERATIONS DURING ABVD CHEMOTHERAPY: CORRELATION WITH CLINICAL, LABORATORY FEATURES AND PROGNOSTIC SIGNIFICANCE

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Background. Serum CRP levels are elevated in the majority of patients with Hodgkin's lymphoma at diagnosis, reflecting tumor burden and aggressive biologic behaviour. A rapid metabolic response (negative PET-2) is a very strong favourable prognostic factor in advanced disease. CRP alterations might parallel disease activity. However there are very few data regarding the short- and mid-term CRP alterations during chemotherapy and their potential prognostic significance. *Aim.* (1) To analyze the correlation between baseline CRP and clinical-laboratory findings and outcome of HL (2) To evaluate the alterations in CRP levels during ABVD chemotherapy and correlate them with the post-chemotherapy PET findings and failure free survival (FFS). Patients and *methods.* Baseline CRP levels were recorded in 273 patients. Serial CRP measurements prior to ABVD and prior to cycles 1b,2a,3a and 4a were performed in 71 patients. CRP levels at that time-points were correlated with post-chemotherapy PET findings (chi-square test) and FFS (Kaplan-Meier curves, log-rank comparison). CRP levels >5 mg/L were

considered elevated. *Results.* (1) For the 273 patients with baseline CRP available, the median value was 19.7 mg/L; 28% and 72% had normal and elevated CRP levels respectively. Baseline CRP levels correlated with virtually all other parameters reflecting tumor burden or disease aggressiveness. 3-year FFS for patients with CRP levels <5, 5-19.69, 19.7-68.96 and >68.96 mg/L (roughly the CRP quartiles) was 80%, 68%, 58% and 67% (P=0.10). Although the difference was significant for patients with normal *vs.* elevated CRP levels (P=0.01), it was not retained in multivariate analysis. (2) For the 71 patients with serial CRP determinations, the median baseline CRP was 28.1 mg/L; 27% and 73% had normal and elevated CRP levels respectively. The median CRP levels prior to cycles 1b,2a,3a and 4a ranged between 3.16 and 3.30 mg/L. The proportion of patients with normal CRP levels increased from 27% at baseline to 69%, 68%, 79% and 73% prior to cycles 1b,2a,3a and 4a. At the time of the analysis, post-ABVD PET was available in 62 patients: It was negative in 48 patients and positive in 14. There was no correlation between interim CRP normalization at any time and post-ABVD PET findings. Only 6 patients have progressed for a 18-month FFS of 90%. Normal CRP levels prior to cycles 3a and 4a were associated with better 18-month FFS rates (92% *vs.* 83%, P=0.32 and 95% *vs.* 86%, P=0.33 respectively), but differences were small and did not reach statistical significance. Similar results were obtained when the analysis was restricted to patients with elevated baseline CRP levels. *Conclusions.* CRP levels are elevated in ~70% of HL patients at diagnosis but did not correlate with FFS independently from other established prognostic factors in the present series of patients. CRP levels are subjected to very rapid alterations during ABVD chemotherapy, which are already demonstrable by the end of the second week. Our preliminary results did not reveal differences in short-term FFS, large enough to be statistically significant. However, analysis of larger number of patients and extension of follow-up are needed.

0888

SEROPOSITIVITY FOR HUMAN CYTOMEGALOVIRUS IS ASSOCIATED WITH BOTH EBV-NEGATIVE AND EBV-POSITIVE CLASSICAL HODGKIN LYMPHOMA

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Background. Classical Hodgkin Lymphoma (cHL) is associated with Epstein-Barr Virus (EBV) in a proportion of cases. Risk of EBV-associated cHL increases with age. EBV persistently infects the majority of healthy adults and the virus is normally controlled by the cytotoxic T-lymphocyte (CTL) response. Cytomegalovirus (CMV) is also associated with persistent infection in ~60%-90% of adults. Control similarly relies on CTL responses. In infected healthy adults, much of the T-cell response is CMV restricted, 10-50% over 60 years of age. Evidence is emerging for an association between CMV infection and immune senescence. The presence of CMV has been shown to impair the CTL response to EBV. The increased proportion of the CTL response directed toward CMV with increasing age may hinder the ability of the remaining T-cell population to respond to other challenges, leading to subtle immune suppression. *Aims.* We investigated the a priori hypothesis that the increased proportion of the immune response diverted to CMV would reduce immunity to EBV, thus increasing the risk of EBV-positive cHL in CMV+ve individuals. *Methods.* Serum was available from 549 cases and 396 controls. EBV status of cHL was available for 491 cases. Ethical approval had been granted. CMV serotyping was performed using the Abbot CMV IgG chemiluminescent microparticle immunoassay. CMV seropositivity was compared in: cHL cases *vs.* controls; EBV-positive cHL cases *vs.* controls; EBV-negative cHL cases *vs.* controls; and EBV-positive cHL cases *vs.* EBV-negative cHL cases using logistic regression. *Results.* 51.1% of all subjects were CMV+ve. As expected, incidence of CMV seropositivity increased with age (15-34 years 33.7%, 35-49 years 49.8%, >50years 73.6%.) After correcting for age and sex, cHL was independently associated with CMV seropositivity (odds ratio (OR) cases *vs.* controls=1.4, 95% confidence interval (CI) 1.05-1.86, P=0.022.). EBV-positive cHL was associated with CMV seropositivity (OR EBV-positive cases *vs.* controls=1.59 95% CI 1.06-2.46, P=0.026.) This association was also seen in EBV negative cases (OR 1.407, 95% CI 1.015-1.949, P=0.040.) There was no difference in CMV seropositivity between EBV-positive and negative cHL cases after adjusting for the effect of age. In order to exclude the possibility that increased nosocomial exposure accounted for the increased seroprevalence in cases, we repeated the analysis using specimens obtained at the time of diagnosis (n=105). The association of cHL with CMV seropositivity was maintained (OR 1.61, 95% CI 1.01- 2.57 P=0.045.) The seroprevalence was not significantly different between pre-treatment (52.4%) and follow-up specimens obtained >200 days post diagnosis (51.2%.) Following stratification by age, the increased odds of cHL associated with CMV seropositivity was observed only in adults >50 years (OR 1.76, 95% CI 1.08- 2.85, P=0.022.) Case:control ORs were greatest for EBV-positive cHL cases (OR 2.37, 95% CI 1.16-4.82, P=0.017.) *Summary/Conclusions.* Classical HL is associated with CMV seropositivity. This effect was observed in EBV-positive and EBV-negative cHL cases and was greatest in older adults. Among older adults, CMV-seropositive individuals had a 2.4-fold increased odds of EBV-positive cHL. This observation supports the hypothesis that reduced immune surveillance due to CMV infection leads to an increased risk of cHL.

0889

A NEW APPROACH TO LONG TERM FOLLOW UP OF LYMPHOMA PATIENTS: TRANSFER TO THE PRIMARY CARE SETTING

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Background. The follow up of patients more than 5 years from completion of treatment for curable lymphomas is largely aimed at the detection of late effects of treatment as the risk of relapse decreases. Since follow up appointments usually do not coincide with significant events, we began to question the appropriateness, effectiveness and safety of maintaining annual long term follow-up for this particular patient population within our tertiary centre. *Aim.* We decided to initiate a policy of patient education regarding possible late effects with a view to transferring care of certain patients on long term follow up to their primary care physicians. *Methods.* Patients ≥5 years since completion of treatment for curable lymphomas (Hodgkins and high grade) were considered potentially suitable for this policy. Patients who had undergone high dose therapy with stem cell support were excluded. A proforma was devised, with primary care physician input, to facilitate discussion of prior treatment(s) and potential late effects with patients in clinic. Information recorded included histological diagnosis and a detailed history of all previous treatments. Risks discussed with all patients were: relapse, secondary malignancies, (including breast cancer in patients who had received mantle radiotherapy), sub fertility and early menopause. Cardiovascular and pulmonary toxicities, hypothyroidism and infection post splenectomy/splenic irradiation were discussed, depending on previous treatments, allowing consultation tailored to the individual patient. Emphasis was placed on developing an awareness and early recognition of symptoms and signs of late effects or recurrence. Lifestyle and diet, particularly geared towards cardiovascular risks, were also discussed. Verbal information was supplemented by a written patient information leaflet. A detailed summary was sent to their general practitioner and copies offered to patients. The medical or clinical nurse specialists were able to initiate the discharge plan. *Results.* 100 patients have been discharged from the lymphoma clinic to date. 3 patients (3%) preferred to continue follow up in the specialist centre. The remaining 97% were comfortable with follow up being transferred to primary care. 6 patients (6%) were re-referred and seen promptly. Two are undergoing investigations for suspected relapse. Two were confirmed relapse. One is being monitored. One presented with pericardial effusion as the first indication of a radiation induced sarcoma. *Summary/Conclusions.* The implementation of a new policy of long-term follow up patients with curable lymphomas has been well received by patients (97%) and has led to streamlining our busy clinical service. Those patients within this new system who have needed urgent review following discharge have received timely re-introduction into the service (6%). This change in approach to routine follow-up care has identified an emerging need for the introduction of 'health and well being' clinics at the end of patients' treatments. The implementation of such a clinic is underway.

0890

CLINICAL FEATURES AND OUTCOMES IN PATIENTS WITH RELAPSED HODGKIN LYMPHOMA RECEIVING SALVAGE CHEMOTHERAPY: DATA FROM CONSORTIUM FOR IMPROVING SURVIVAL OF LYMPHOMA (CISL) IN KOREA

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Background. Hodgkin's lymphoma (HL) is a potentially curable disease in at least 80% of patients. However, up to a 10% of patients do not achieve a complete response (CR) with first line therapy and 10~40% of CR patients suffer a relapse. HL is an uncommon disease, especially rare in Korea, around 5% of all lymphoma. *Aims.* In this current study clinical features and outcomes of relapsed HL in Korea were evaluated. *Methods.* Between January 1984 and October 2009 clinical data were collected retrospectively from 15 centers in Korea. *Results.* Four hundred and ninety two patients with newly diagnosed HL were included in this study and 425 of entire patients were adequate for response analysis. After first line therapy, 375 patients (88%) exhibited CR. The CR rate was 93.3% in the radiotherapy group, 82.1% in chemotherapy group, and 76.3% in combined chemo-radiotherapy group. Twenty patients (4.7%, 20/425) were refractory to initial therapy. A total of 105 (28%) of 375 patients who had achieved CR relapsed. Clinical characteristics including no palpable mass, presence of B symptoms, ECOG performance status (2 or 3), and stages (3 or 4) were significantly associated with relapse after achieving CR with initial therapy. Seventy five patients received salvage chemotherapy with various regimens including DHAP (18%), ESHAP (11%), ABVD (11%), C-MOPP (7%) or others. Of the 75 patients who received salvage therapy, 60 patients achieved 80% response, including 59% CR (n = 44), 21% partial response (PR) (n = 16), and 20% progressive disease (PD) (n = 15). The salvage regimens were classified in two groups according to whether the regimens contained cisplatin. The outcome of salvage therapy was evaluated. There was no significant difference in response rate between two groups (cisplatin containing regimens 58.8%, non-cisplatin containing regimens 82.4%, unknown 71.7%, P=0.3). Twenty one patients received high-dose chemotherapy (HDT) followed by autologous stem cell transplantation (ASCT). Of the 21 patients who completed HDT followed by ASCT, 12 patients (57.1%) exhibited CR, 2 patients (9.5%) exhibited PR, 1 patient (4.8%) experienced stable disease (SD), and 6 patients (28.6%) experienced refractory disease. *Conclusions.* Relapse rate in patients with HL in Korea was similar to that in western people. A majority of relapsed patients could be salvaged with chemotherapy and/or HDT followed by ASCT. Both cisplatin containing or non-cisplatin containing regimens were reasonable option as a salvage treatment. An optimal regimen needs to be defined with prospective trials.

0891

FIRST-LINE CHEMOTHERAPY LEADS TO HIGH BONE TURNOVER AND REDUCED BONE MASS IN PATIENTS WITH NON-HODGKIN'S LYMPHOMA: RESULTS FROM A PROSPECTIVE STUDY

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Background. Chemotherapy-associated osteoporosis increases the risk for fractures and deteriorates quality of life in several malignancies. There are very limited data for the effect of chemotherapy on bone metabolism of adult patients with non-Hodgkin's lymphoma (NHL). *Aim.* The aim of this prospective study was to evaluate bone remodeling in NHL patients, pre- and post- first-line chemotherapy. *Patients/Methods.* As of February 2010, 60 patients were enrolled in this study and 43 (26M/17F, median age 60 years, range: 18-90 years) had completed first-line chemotherapy. Twenty-eight patients (65%) had diffuse large B-cell lymphoma, 5 (12%) follicular lymphoma (grade III), 4 (9%) mantle-cell lymphoma, 4 marginal-zone lymphoma and 2 T-cell NHL. Sixteen (37%) had stage IV disease and 19 (44%) had B-symptoms before therapy. Thirty-nine patients (91%) received R-CHOP, 2 R-COP and 2 CHOP as first-line therapy. Bone mineral density (BMD) of the lumbar spine (L1-L4, antero-posterior view), and femoral neck (FN) was measured by DXA on day 1 of cycle 1 (baseline) and on day 30 post the last cycle of chemotherapy. The following serum indices of bone metabolism were measured on the days of DXA: i) osteoclast stimulators (sRANKL and osteoprotegerin), ii) osteoblast regulators [PTH, vitamin-D and dickkopf-1 (Dkk-1)], iii) bone resorption markers (CTX and TRACP-5b) and iv) bone formation markers [bone alkaline phosphatase (bALP) and osteocalcin]. The above markers were also evaluated in 24 gender- and age-matched controls. Patients were followed for skeletal-related events (SREs) throughout the period of the study. *Results.* At baseline, NHL patients had a median T-score of L1-L4 BMD of -0.59 (range -4.27 to +3.68) and of FN BMD of -1.02 (-4.01 to +1.67). The median T-score of the lumbar vertebra with the major bone loss (LVMBL) was -1.41 (-4.6 to +3.03). There was a strong correlation between L1-L4 and FN BMD (r=0.687, P<0.0001), between L1-L4 BMD and Dkk-1 (r=-0.62, P<0.0001) and between CTX with TRACP-5b (r=0.463, P<0.0001) and sRANKL (r=0.386, P=0.004). The administration of chemotherapy resulted in a dramatic reduction of BMD both in L1-L4 and FN (L1-L4 median T-score: -1.12; range -4.49 to +3.04; P<0.0001, LVMBL median T-score: -1.41; range: -4.6 to +3.03; P=0.002, FN median T-score: -1.16; range: -3.68 to +1.12; P<0.0001) compared to baseline values. The reduction of L1-L4 and FN BMD post-chemotherapy was more profound in males (P=0.009 and P=0.009 respectively) than in females (P=0.023, P=0.013) and in patients of >55 years (P=0.001 and P=0.003, respectively) compared to all others (P=0.094, P=0.03). Patients who received 8 cycles of chemotherapy had a greater reduction of L1-L4 (P=0.001) and FN (P=0.006) BMD compared to all others. CTX (P=0.031) as well as both markers of bone formation, bALP (P<0.0001) and osteocalcin (P<0.0001) were significantly increased post chemotherapy. During study period, one patient had a pathological fracture in his right FN. *Summary/Conclusions.* Our on-going study suggests that first-line chemotherapy results in high bone turnover, which leads to increased bone loss and reduced BMD of L1-L4 and FN in NHL patients. The prophylactic use of anti-resorptive agents, such as bisphosphonates, may be useful in these patients.

0892

SAFETY AND EFFICACY OF BENDAMUSTINE WITH OR WITHOUT RITUXIMAB IN THE TREATMENT OF HEAVILY PRETREATED LYMPHOMA PATIENTS. A MULTICENTER RETROSPECTIVE STUDY.

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Background. Bendamustine is an alkylating agent with a nitrogen mustard group and a purine like benzimidazol group. Recently this drug was introduced in Italy and it was used in patients pretreated to test its efficacy and safety. *Aims.* The aim of this study was to collect all the Italian experiences to evaluate the results in term of response to therapy and hematological and extra-hematological toxicities. *Methods.* We analized lymphoma patients treated in twenty-four hematological Italian centers with Bendamustine alone or in combination with anti-CD20 antibody. Patients who have received at least one complete cycle were evaluated for response and toxicity. The treatments consisted of: Bendamustine 60-90 mg/m^2 days 2,3 alone or in combination with Rituximab 375 mg/m^2 day 1, every 21 or 28 days. *Results.* One-hundred sixty-eight patients were analized, median age was 69 (range 26-87), 103 were male and 65 female, the diagnosis were: 61 indolent non-follicu-

lar lymphoma (small lymphocitic, marginal, lymphoplasmocitic), 34 diffuse large B cell lymphoma, 44 follicular lymphoma, 29 mantle cell lymphoma, 3 Peripheral T cell lymphoma. Patients were heavily pretreated the median number of previous treatments was 3 (range 1-9), 60 patients have experienced more than three chemotherapy schemes. One-hundred twenty-nine patients were previously treated with Rituximab and 21 have performed an autologous transplantation. The Bendamustine pre-treatment condition was: 72 relapsed patients, 35 with refractory disease and 60 with a progressive disease after partial response. The median number of Bendamustine cycles was 4 (range 1-11). All patients were evaluable for response: 49 (29%) complete remission, 72 (43%) partial response or stable disease with an overall response rate of 72% and 47 non responders. No differences were observed according to Bendamustine dosage or scheduling. According to histotype we observed that 20/29 mantle cell lymphoma (11 CR and 9 PR) obtained a response to therapy, 48/58 (13 CR and 35 PR) indolent non follicular lymphoma, 42/44 (21 Cr and 21 PR) follicular lymphoma obtained a response and 11/34 (4 CR and 7 PR) DLBCL obtained a response. With a median period of observation of 8 months (1-36) 70% of patients are alive. In this group of heavily pretreated patients 685 cycles were performed: the extrahematological toxicity was really mild and the hematological toxicity was trombocytopenia grade 3-4 in 16 patients (9%) and neutropenia grade 3-4 in 29 patients (17%). *Conclusion.* In summary this retrospective study shows that treatment with Bendamustine alone or in combination with Rituximab is a safe and efficacy regimen in a subset of pluriresistent patients. This data shows also that the best results could be obtained in indolent lymphoma and incouraging data in mantle cell lymphoma.

0893

A PHASE I TRIAL OF BTK INHIBITOR PCI-32765 IN PATIENTS WITH RELAPSED NON-HODGKIN'S LYMPHOMA: EVIDENCE OF ANTITUMOR ACTIVITY

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Background. A functional B cell receptor is maintained in the majority of B-cell malignancies. PCI-32765 is a novel oral agent that has been shown to selectively inhibit Bruton's tyrosine kinase (Btk), a downstream mediator of B-cell receptor signaling. *Aims.* This single-arm, multicenter, phase I study was conducted to determine the safety and tolerability of PCI-32765 in patients with relapsed/refractory non-Hodgkin's lymphoma. *Methods.* Adult patients with relapsed or refractory B-cell lymphoma were eligible for trial entry. Cohort I was dosed at 1.25 mg/kg/day, with subsequent dose escalation (2.5, 5.0, 8.3, 12.5, 17.5 mg/kg) based on safety evaluation. Adverse events (AEs) were graded using the CTCAE v3.0 and by laboratory assessment of hematologic toxicity. In the absence of DLTs, dose escalation was planned to proceed to three dose levels above the cohort that achieved >90% occupancy of Btk by PCI-32765 assessed using a PD probe assay. Blood was collected before and after treatment on Days 1 and 8 for determination of pharmacokinetic and pharmacodynamic (PD) profiles and clinical responses were assessed every two cycles. *Results.* 30 pts (11 follicular lymphoma (FL), 7 CLL/SLL, 5 DLBCL, 4 mantle cell lymphoma (MCL), 3 marginal zone lymphoma) with a median of 3 prior therapies have been enrolled on four cohorts (1.25, 2.5, 5.0 and 8.3 mg/kg/day). Therapy was well tolerated with most adverse events < grade 2. Two protocol defined DLTs have been observed. 1 patient required dose delay > 7d due to neutropenia, and 1 patient developed hypersensitivity reaction to study drug. 19 patients are currently evaluable for response. The ORR is 42%; 1 CR (SLL), 7 PR (4 CLL/SLL, 2 MCL and 1 FL). In cohort 2, PD assays demonstrate complete occupancy of Btk by PCI-32765, with >95% enzyme occupancy 4 hours post dose in all pts. Basophil degranulation, a Btk-dependent cellular process, was completely inhibited up to 24 hrs. T-cell responses were not affected, and no significant depletion of peripheral blood B, T or NK cell counts observed. Plasma concentrations of PCI-32765 generally increased with increasing dose. Trough levels of PCI-32765 did not increase meaningfully with repeated dosing. Analysis of pharmacokinetic and pharmacodynamic profiles on Day 1 showed that Btk active-site occupancy was saturated 4 and 24 hours postdose at AUC values of ≥ 200 ng-h/mL. At steady state all patients that received doses of greater than 1.25 mg/kg/day had AUC values ≥245 ng-h/mL. *Conclusion.* PCI-32765 is an oral agent that selectively inhibits Btk at low dose levels. Monotherapy appears to be well tolerated in patients with relapsed B-cell NHL with encouraging clinical activity and a mild toxicity profile. Toxicity and response assessment is ongoing. Phase II trials in indolent lymphoma and MCL are planned.

0894

PHASE 1 STUDY OF NAVITOCLAX (ABT-263) PLUS RITUXIMAB IN CD20-POSITIVE LYMPHOID MALIGNANCIES

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Background. Navitoclax (ABT-263) is a novel, orally bioavailable, small molecule that binds with high affinity (Ki ≤1nM) to Bcl-2, Bcl-xL, and Bcl-w, promoting apoptosis. *in vitro*, navitoclax shows potent targeted cytotoxicity (EC_{50} ≤1μM) against T and B lymphoid malignancies that over-express Bcl-2. Phase 1 monotherapy results show that oral navitoclax is well-tolerated and with daily dosing has activity in some patients (pts) with lymphoid malignancies and many pts with chronic lymphocytic leukemia (CLL). Thrombocytopenia is a dose-limiting toxicity (DLT). Rituximab is an established treatment for pts with low-grade, CD20-positive B-cell malignancies, as monotherapy or in combination with chemotherapy. Navitoclax showed enhancement of rituximab efficacy in multiple preclinical models of B-cell lymphoma, both with rituximab monotherapy and in combination with chemotherapy. *Aims.* The primary objective of this, international, phase 1b/2 dose-escalation study is to assess safety and compatibility of oral navitoclax added to standard rituximab monotherapy in pts with relapsed/refractory B-cell malignancies. Secondary objectives include navitoclax pharmacokinetics (PK) and maximum tolerated dose (MTD) determination for the combination. *Methods.* Key eligibility includes presence of ≥1 lesion ≥1.5 cm, ECOG score ≤1, and platelet count of >75,000/uL. Following a 7-14 day 150 mg/day dose lead-in, navitoclax was dosed at 200mg or 250mg per day continuously. Patients received 375mg/m^2 rituximab once weekly for 4 doses, commencing after 7 days of navitoclax. A cycle was defined as 28 days of therapy. Tumor responses were evaluated using IWG criteria and NCI-WG criteria (for CLL pts) every 2 months. Patients may continue on navitoclax therapy for 2 years in the absence of progressive disease or significant toxicity. *Results.* As of February 1, 2010, 11 pts (5 with follicular lymphoma [FL], 1 with B-lymphoblastic lymphoma, 1 with CLL, 1 with DLBCL, 1 with transformed disease (DLBCL), 1 with SLL, 1 with lymphoplasmacytic lymphoma), median age 60 years (range 46-79), have been dosed with navitoclax and rituximab (4 in the 200 mg; 7 in the 250mg cohort). Informed consent was obtained. One pt in the 250mg cohort had a DLT of Grade 3 diarrhea. Two pts (1 with B-lymphoblastic lymphoma, 1 with DLBCL) discontinued due to disease progression, and 1 pt withdrew consent. Eight pts remain on study: 2 in the 200mg cohort, 6 in the 250mg cohort. Preliminary efficacy data indicate 1 PR (FL 189+ days) and 1 SD (CLL 176+ days) in the 200 mg cohort, and 2 PRs in the 250mg cohort (1 FL 126+ days, and 1 SLL 111+ days). Based on the limited data, PK of navitoclax in this combination study appears to be comparable to PK in the navitoclax monotherapy study. *Summary/Conclusions.* The combination of navitoclax and rituximab is well tolerated and shows preliminary evidence of activity. The MTD has not been reached. Accrual is ongoing and following completion of the dose-escalation component of this study, an expanded cohort of pts will be assessed using the recommended phase 2 dose of navitoclax to estimate the objective response rate of this combination.

0895

THE CLINICAL UTILITY OF FDG PET-CT IN EVALUATION OF BONE MARROW INVOLVEMENT BY LYMPHOMA

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Background. In lymphoma, bone marrow (BM) involvement is a sign of extensive disease and BM biopsy is a standard method in the evaluation of BM infiltration. Because of focal BM involvement pattern by

lymphoma, the reported rates of unilateral involvement in bilateral biopsies range from 10% to 50%. However BM biopsy is an invasive and painful procedure. The value of unilateral *vs.* bilateral BM biopsy remains controversial. Fluorodeoxyglucose positron emission tomography-computed tomography (FDG PET-CT) is a noninvasive imaging technique and currently it shows potential to detect BM involvement by lymphoma. *Aims.* We assess the abilities of FDG PET-CT to ascertain the presence of BM involvement and to define the patients whose bilateral BM biopsies could be replaced by unilateral biopsy in lymphoma. *Methods.* We retrospectively reviewed medical records of histologically proven lymphoma patients from 2004 through 2009 at the Hallym University Medical Center. All patients were examined by FDG PET-CT and iliac crest BM biopsy for initial staging work-up. We used both qualitative and quantitative methods to evaluate FDG PET-CT. All lesions in iliac crest were analyzed by maximum standardized uptake value (SUVmax) and cut-off value for positivity was 2.0 g/mL. We calculated sensitivity, specificity, positive predictive value and negative predictive value for FDG PET-CT in evaluation of BM infiltration, using BM biopsy as the reference standard. *Results.* Study population comprised 101 consecutive patients (median age 58.4, range 20-86 years ; 62 male) with Hodgkin's disease (n=8) or non-Hodgkin's lymphoma (NHL, total, n=93; diffuse large B cell, n=56; angioimmunoblastic T cell, n=10; marginal zone B cell, n=8; NK/T-cell, n=7; peripheral T cell, n=3; Mantle cell, n=3; Anaplastic large cell, n=3; Small lymphocytic, n=2; Follicular, n=1). Overall, BM involvement that confirmed by BM biopsy occurs in 25 patients. SUVmax values on iliac crest of patients with lymphoma infiltrated BM were significantly higher than those of patients with intact BM (2.97±1.83 *vs.* 1.30±0.35 g/mL; $P < 0.0001$). Table1 shows the calculated values for FDG PET-CT in evaluation of BM infiltration which had been measured by quantitative method. The values measured by qualitative method were similar (sensitivity 42%, specificity 99%, positive predictive value 92%, negative predictive value 83%). There was no significant difference in the ability of the FDG PET-CT detecting BM involvement among Hodgkin's lymphoma, Indolent NHL and aggressive NHL. *Conclusions.* This study demonstrates good concordance of FDG PET-CT with the results of BM biopsy for the detection of BM infiltration in lymphoma patients. Because of the excellent positive predictive value, FDG PET-CT will be a useful tool for image-guided biopsy for lymphoma staging. And in lymphoma patients with increased iliac crest FDG uptake, unilateral BM biopsy could be substituted for bilateral BM biopsy.

Table. FDG PET-CT and Bone marrow involvement result.

	Bone marrow involvement (%)			
	HD	Indolent NHL	Aggressive NHL	Total
Sensitivity	50 (1/2)	46 (6/13)	50 (5/10)	48 (12/25)
Specificity	100 (6/6)	100 (11/11)	98 (58/59)	99 (75/76)
PPV	100 (1/1)	100 (6/6)	83 (5/6)	92 (12/13)
NPV	86 (6/7)	61 (11/18)	92 (58/63)	85 (75/88)

PPV: positive predictive value; NPV: negative predictive value; HD: Hodgkin's disease; NHL: non-Hodgkin's lymphoma; FDG PET-CT: Fluorodeoxyglucose positron emission tomography-computed tomography

0896

ANEMIA AND ESA ADMINISTRATION IN PATIENTS TREATED WITH RCHOP CHEMOTHERAPY: RESULTS FROM AN OBSERVATIONAL STUDY OF PATIENTS WITH NON-HODGKIN LYMPHOMA

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Background. The CHOP (cyclophosphamide, doxorubicin, vincristine, prednisolone) regimen and its variants are regarded as the standard of care for treating aggressive advanced-stage non-Hodgkin lymphoma (NHL). Anemia is a common side effect of myelosuppressive chemotherapy (CT), and anemia greater than grade 2 (WHO definition; hemoglobin (Hb) <8 g/dL) has been found in 25% of patients receiving RCHOP chemotherapy. Erythropoiesis-stimulating agents (ESAs) are approved for the treatment of CT-induced anemia to raise Hb concentrations and reduce the need for blood transfusions. *Aims.* In this primary analysis from the IMPACT NHL study, we aimed to describe predictors of anemia, current treatment practices of ESA administration, and efficacy outcomes relating to hemoglobin (Hb) levels in NHL patients treated with ESAs. *Methods.* IMPACT NHL was a retrospective and prospective multi-center observational study in Europe and Australia. NHL patients (≥18 years) were enrolled between 2005 and 2008 and received either CHOP-14 or CHOP-21 chemotherapy±rituximab (R). Data were collected on patients who were treated with ESAs (darbepoetin-α, epoetin-α, or epoetin-β) according to routine clinical practice, at any time during the study. The analysis reported here presents the results of pre-specified anemia-related outcomes. Anemia was defined as Hb <10 gdL. Multivariate logistic regression was used to explore predictors of anemia during chemotherapy treatment (CT). *Results.* A total of 1829 patients treated with at least one cycle of CHOP (± R) chemotherapy were enrolled in the study. Of the patients with available Hb values (n=1687 at start of CT treatment; n=1810 during CT treatment), 11% were anemic at the start of CT treatment, while 33% were anemic during CT treatment. Significant predictors of anemia were older age, higher number of chemotherapy cycles, lower Hb at start of CT treatment, female gender, Ann Arbor Stage IV, CHOP-14 (± R) chemotherapy, and diffuse large B-cell lymphoma. During the study 404 patients (22%) received ESA treatment (see Table for baseline characteristics). Two thirds of these patients initiated their ESA treatment within the first three cycles of CT, and most frequently in response to low Hb concentrations (81%). Mean Hb value at initiation of ESA treatment was 10.07 g/dL (SD 1.28): 45% of patients had Hb <10 g/dL, 15% Hb <9 g/dL and 63% had Hb 9-11 g/dL (per EORTC guidelines). After initiation of ESA treatment, Hb concentrations increased regardless of the CT cycle. Of 181 patients with Hb <10 g/dL at ESA initiation, 57% achieved Hb 10-12 g/dL during the ESA treatment phase (Kaplan-Meier (KM) estimate 88%; 95% CI 79, 97). *Conclusions.* In this observational study, 33% of patients were anemic during CT treatment. Twenty-two percent (22%) were treated with ESAs, most frequently in response to low Hb concentrations. In ESA treated patients, 2/3 of patients were started on ESA treatment according to EORTC guidelines (Hb 9-11 g/dL), and 88% (KM %) of ESA-treated patients with Hb <10 g/dL at ESA initiation achieved Hb concentrations within the target range of 10-12 g/dL.

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Table.

n (%)	ESA Treatment (N=404)	No ESA Treatment (N=1425)	Total (N=1829)
Ann Arbor Stage			
I-III	172 (43)	895 (63)	1067 (58)
IV	231 (57)	525 (37)	756 (41)
missing	1 (0)	5 (0)	6 (0)
ECOG status			
0-2	366 (91)	1334 (94)	1700 (93)
3-4	16 (4)	26 (2)	42 (2)
missing	22 (5)	65 (5)	87 (5)
Histology			
Diffuse large B cell	269 (67)	867 (61)	1136 (62)
Follicular	65 (16)	280 (20)	345 (19)
Other	70 (17)	276 (19)	346 (19)
CT Regimen			
CHOP-14	12 (3)	37 (3)	49 (3)
RCHOP-14	111 (27)	376 (26)	487 (27)
CHOP-21	17 (4)	65 (5)	82 (4)
RCHOP-21	264 (65)	947 (66)	1211 (66)

0897

STANDARDIZED UPTAKE VALUE OF 18F-FDG ON PET/CT IN NEWLY DIAGNOSED PATIENTS WITH NON-HODGKIN'S LYMPHOMA CORRELATES WITH PROLIFERATIVE INDEX OF LYMPHOMA CELLS

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Background. Positron emission tomography integrated with computed tomography (PET/CT) using 2-[fluorine-18]-fluoro-2-deoxy-D-glucose (18F-FDG) is currently considered to be the most beneficial imaging method for staging patients with non-Hodgkin's lymphoma (NHL). The intensity of 18F-FDG accumulation may be semiquantitatively determined by calculating the so-called standardized uptake value (SUV). *Aims.* The prospective study aimed at assessing the benefit of SUVmax determination in staging 18F-FDG PET/CT in 149 untreated patients with NHL, and comparing the SUVmax values with the Ki-67 proliferative index of tumor cells and serum lactate dehydrogenase (LDH) levels. The prospective study was approved by the Multicenter Ethical Committee of the Faculty of Medicine and Dentistry and University Hospital Olomouc. The patients gave written informed consent to examination and anonymous data analysis. *Methods.* For each patient, SUVmax of the suspicious lesion was calculated. This was based on values measured in the area of interest around the pathological lesion seen in the PET scan as a pixel with the highest accumulation of 18F-FDG. For this purpose, the longer axis of each evaluated lymph node or organ lesion had to be longer than 20 mm. *Results.* In the entire group of patients, the geometric mean of SUVmax values was 10.2 (95% CI; 9.1 - 11.5). The highest mean and median values of SUVmax were observed in patients with diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL) and peripheral T-cell lymphoma (PTCL); the lowest mean and median values were found in mantle cell lymphoma (MCL), marginal zone lymphoma (MZL), and small lymphocytic lymphoma / chronic lymphocytic leukemia (SLL/CLL). Statistically significant differences in SUVmax values ($P<0.001$) were found between patients with DLBCL and those with MCL, DLBCL and MZL. The overlap of SUVmax between DLBCL, FL and PTCL was very significant. A similar overlap in SUVmax < 10 was found between DLBCL and the other subgroups of NHL. Statistically, no correlation was found between the LDH and SUVmax values. On the other hand, a correlation of the Ki-67 proliferative index of tumor cells and SUVmax was revealed ($r=0.409$, $P<0.001$). In patients with Ki-67 ≤ 60, the geometric mean of SUVmax was 8.8 (95% CI; 7.3 - 10.5) and the median was 9.4 (range; 2.0-43.2). In patients with Ki-67 > 60, the geometric mean of SUVmax was 14.3 (95% CI; 12.2-16.8) and the median was 15.7 (range; 2.2-40.6). *Conclusions.* The study results confirm that SUVmax determined during initial staging 18F-FDG PET/CT is not beneficial for making a more precise diagnosis in most patients with NHL. Correlation of SUVmax with the Ki-67 values suggests that SUVmax might have a prognostic values in certain NHL subtypes.

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0898

PREVALENCE OF CHRONIC INFLAMMATORY DISORDERS IN PATIENTS WITH MALIGNANT LYMPHOMA AT DIAGNOSIS

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Background. There is evidence for the role of chronic antigenic stimulation (CS) in the development of cancer. However, clinical data are rare as is information on how the presence of a chronic inflammatory disease influences outcome. *Aims.* To assess the presence of chronic infections and autoimmune diseases at diagnosis in patients with malignant lymphoma and to investigate the association of these data with basic clinical information and survival. *Methods.* Patient charts were reviewed to assess the presence of chronic infections and autoimmune diseases in lymphoma patients. Data collected included sex, age, age at diagnosis, mortality, lymphoma subtype, and treatment. Data analysis sought to detect correlations between patient characteristics and absence, presence, and type of chronic inflammatory disorder, respectively. *Results.* In this retrospective, monocentric study, 367 patients diagnosed with malignant lymphoma at our institution between 2001 and 2005 were included. Of these patients, 297 suffered from non-Hodgkin´s lymphoma (NHL) and 70 from Hodgkin´s lymphoma (HL). In total, 9.8% (N=36) had a history of chronic antigenic stimulation (4.4% autoimmune diseases, 5.4% chronic infections). After a median observation time of 74.7 months (quartiles: 62.6 to 89.1 months), 118 patients have died (32.2%). Interestingly, CS patients had a slightly better performance than non-CS patients, although no significant difference in overall survival could be observed (Kaplan-Meier estimates for survival after 48 months in HL: CS 100%, non-CS 88.7%; aggressive NHL: CS 66.7%, non-CS 58.6%; indolent NHL: CS 84.6%, non-CS 77.0%). Slightly more men than women had malignant lymphoma (52.2% *vs.* 47.8%), both in the entire patient cohort and in NHL and HL subgroups. However, in all groups sex prevalence among patients showing chronic antigenic stimulation was skewed in favor of women where they constituted over 60% of patients ($P=0.018$). Women were in particular overrepresented among lymphoma patients with autoimmune diseases ($P=0.001$). Patients with chronic inflammatory conditions were more likely to suffer from an aggressive lymphoma ($P=0.256$). In particular, NHL patients with autoimmune diseases showed a tendency for diffuse large B cell lymphoma (NHL+AI 8/12 (66.7%), NHL-AI 107/285 (37.5%); $P=0.066$). Among patients with T-cell lymphoma and lymphoplasmocytic lymphoma, no history of chronic antigenic stimulation could be observed. *Conclusions.* The pre-existence of chronic antigenic stimulation appears to be associated with a higher risk of aggressive lymphoma and a higher prevalence of women. However, in our study we did not observe a difference in overall survival between CS and non-CS patients in the respective lymphoma subgroups. This suggests that in the presence of a chronic inflammatory condition no special treatment strategies are required.

0899

CLINICAL EXPERIENCE OF BENDAMUSTINE TREATMENT FOR NON-HODGKIN LYMPHOMA AND CHRONIC LYMPHOCYTIC LEUKEMIA: SPANISH REGISTRY

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Introduction. Bendamustine (B) is a purine analog/alkylator hybrid that has demonstrated clinical activity in relapsed indolent non-Hodgkin lymphoma (NHL), including those refractory to other alkylating or purine analog agents, chronic lymphocytic leukemia (CLL) and multiple myeloma patients. Bendamustine is currently licensed in Germany and Switzerland for use in NHL and CLL, and is in evaluation process by European Medication Agency (EMEA) in other european countries. *Aim.* To analyze retrospectively the efficacy and toxicity of bendamustine as a single-agent or in combination for NHL and CLL in Spain. *Patients and Methods.* A questionnaire form was sent to Spanish centers in which bendamustine had been used. Patients with relapsed or refractory NHL or CLL after at least 1 prior treatment regimen were eligible. Any bendamustine regimen was included. *Results.* 22 institutions responded favourably for participating in the study and 116 patients (pts) were included in the registry. Median age was 68 years (range: 36-88); male: 61%; ECOG/PS 0-1: 74%; Ann Arbor III-IV: 80% and IPI score >3: 48% in NHL, and Binnet B-C stage in CLL: 66%. Histology was as follows: 47 pts CLL; 20 pts aggressive NHL (16 mantle cell lymphoma, 3 diffuse large B-cell lymphoma and 1 T cell lymphoma); 49 pts indolent NHL (41 follicular lymphoma, 5 extranodal marginal zone B-cell lymphoma of MALT type and 3 lymphoplasmocytic lymphoma); Median time from diagnosis to Bendamustine treatment was 4.9 years (range 1-19) and median number of previous treatment regimens was 3 (range 1-11). 46 pts (41%) were refractory to prior treatment. The most frequent used regimen was rituximab plus B (RB) independently of the histology (see Table 1). No case of Stevens-Johnson syndrome has been recorded. Median number of bendamustine cycles was 4 (range 1-7). 441 cycles of B were administered. 63% of de pts had adverse events grade III/IV, being hematologic toxicity the most frequent adverse event grade III/IV (33% leucopenia, 53% neutropenia, 28% thrombocytopenia, and 20% anemia). 14% cycles of B required pts admission in hospital. 101 pts were assessable for response at the time of analysis. Response rate is showed in Table 1. 39 pts died (22 progression, 9 infection, 6 infection plus progression, 1 acute renal failure and 1 isquemia). *Conclusions.* 1. Bendamustine, at dose of 90 mg/m^2/day for two consecutive days, associated with rituximab was the most common regimen. 2. Bendamustine containing therapy achieved a high response rate in this heavily pretreated population of CLL and NHL patients. Responses were seen in indolent and aggressive histologies, and also in patients with chemoresistant disease to the immediately previous regimen. 3. Treatment with bendamustine was associated with an acceptable toxicity profile in this population of patients.

Table 1.

Total cohort	*Indolent NHL*	*Mantle lymphoma*	*CLL*
N (pts)	116	49	16	47
B mean dose (mg/m^2)	84	86	88	79
More frequent B regimen:				
RB (%)	60	52	90	60
Overall response (%)	63	70	80	49
CR+ncCR (%)	31	34	60	11
N° pat with adverse events grade III/IV	63	56	56	77

Myelodysplastic Syndromes 2

0900

ELEVATED CD44 SERUM LEVELS REPRESENT AN INDEPENDENT PROGNOSTIC FACTOR IN MYELODYSPLASTIC SYNDROMES

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Background. Overexpression of the adhesion molecule CD44 correlates with poor prognosis in various neoplasms. The cleavage of the ectodomain of CD44 results in elevated levels of soluble CD44 (solCD44). SolCD44 retains important biological functions including the regulation of tumor cell proliferation and apoptosis. Aim of this study was to analyse serum levels of solCD44 in a well-defined cohort of MDS patients and to evaluate the relevance of solCD44 in clinical prognostication. *Methods.* Serum levels of soluble CD44 standard (solCD44s) were measured in 130 myelodysplastic syndromes (MDS) patients (median age 68 yrs) using an enzyme-linked immunosorbent assay (ELISA). *Results.* solCD44s levels were significantly elevated in MDS patients as compared to those of healthy donors (P< 0.001) and were found to correlate with distinct MDS subtypes. The highest levels of solCD44s were found in patients with CMML, in RAEB-t and in patients with MDS transformed into secondary acute myeloid leukaemia (AML). In univariate analysis elevated levels of solCD44s (cut-off level > 688.5 ng/mL) correlated significantly with shorter overall survival in MDS patients (12 *vs.* 39 months; P<0.001). In multivariate analysis solCD44s displayed prognostic significance independent of the International Prognosis Scoring System (IPSS). To test for refined prognostication, IPSS risk groups were split into two separate categories based on solCD44s levels. Using this approach, MDS patients with a shorter survival were identified both in the IPSS low-risk (P=0.037) and in the IPSS intermediate-1-risk group (P= 0.015). *Summary/Conclusions.* Elevated sCD44s levels define a cohort of MDS patients with unfavourable prognosis, which might be helpful in risk stratification and in therapeutic algorithms. Understanding of solCD44-mediated processes will offer an important clue toward defining mechanisms responsible for tumour propagation in MDS and AML. Thus, the rationale for therapeutic strategies directed at CD44-mediated interactions will be provided.

0901

ROLE OF RIBOSOMAL GENES IN MYELODYSPLASTIC SYNDROMES EVOLUTION TO ACUTE MYELOID LEUKEMIA

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Background. The haploinsufficiency of the ribosomal protein RPS14 has been involved in the defective erythroid differentiation in the 5q-syndrome. Moreover, a low RPS14 expression has been recently described as a common alteration in other myelodysplastic syndromes (MDS) different to 5q- syndrome, that defines a subgroup of patients with prolonged survival. *Aims.* We aimed to correlate RPS14, RPL28, EE1D ribosomal genes expression, as well as SPARC tumor suppressor gene expression with the different subtypes of MDS, their kariotype, International Prognostic Score System (IPSS), and to determine their role and prognostic value in the evolution to AML. *Methods.* 48 patients diagnosed of MDS (10 with isolated 5q deletion, 7 with chromosomal 7 alterations, and 4 with trisomy in chromosome 8), 10 AML secondary to MDS, 3 myeloproliferative neoplasms (MPN) and 3 controls were included in the present study. Patients were diagnosed and classified according to the WHO criteria. The cDNA was collected from bone marrow aspirate samples. RPS14, RPL28, EE1D and SPARC gene expression was analyzed in duplicate with TaqMan individual assays in an ABI PRISM 7900HT Sequence Detection system (Applied Biosys-

tems). The ratio for each sample was achieved using the comparative Ct method as -2Δct. C18S, ABL and Gus were used as control genes. Statistical significance (P<0.05) was obtained by non-parametric wilcoxon analysis in SPSS15 software. *Results.* The statistical analysis showed a significant decrease in RPS14 (P=0.001) expression in patients with isolated 5q- alteration, compared to the other MDS. On the other hand, RPS14 was significantly decreased in not 5q- MDS compared to AML secondary to MDS (P<0.001), NMP (P<0.001) and controls (P=0.017) (see attached figure). The analysis performed with other clinical variables (gender, SMD type, IPSS, bone marrow blasts percentage and associated cytopenias) showed no significant gene expression differences. *Conclusions.* RPS14 down expression, observed in isolated 5q- syndrome, as previously referred, is a coherent finding, due to the haploinsufficiency of RPS14 in this entity. On the other hand, RPS14 overexpression in AML secondary to MDS could play a role in progression to AML. These results will be confirmed by studies of RPS14 gene expression in a group of patients with AML secondary to MDS, before and after progression to AML.

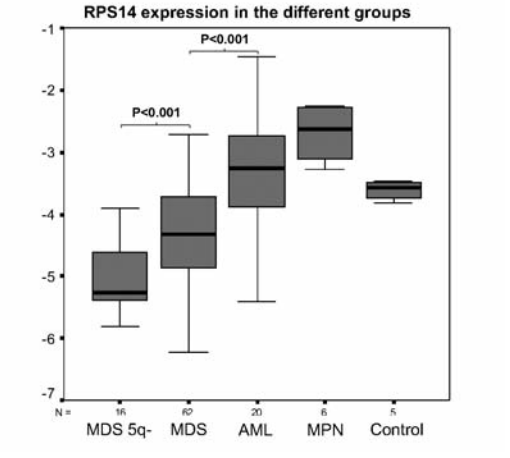

Figure. RPS14 gene expression in the differents groups.

0902

HUMAN TELOMERASE REVERSE TRANSCRIPTASE EXPRESSION (HTERT) DETECTED BY FLOW CYTOMETORY IN CD34 POSITIVE PROGENITOR FRACTION IN MYELODYSPLASTIC SYNDROME AND ACUTE MYELOID LEUKEMIA.

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Telomerase activity (TA) has been found in most common cancers indicating that telomerase detection may be a useful marker in cancer diagnosis. TRAP assay and RT-PCR for hTERT mRNA expression are customarily used for detection of TA, however such methods cannot measure the TA in cell subpopulation. Immunohistochemical detection of hTERT is useful to detect telomerase-positive cells in a background of non- cancerous cells. We developed a method for the detection of intra-nuclear hTERT protein, in a sub-population of hematopoietic cells, using concurrent staining cell surface antigen and multi color flow cytometry hTERT expression detected by flow cytometory were correlated with TA (r=0.0735, P=0.002) (Published in Leukemia Res 2010). We applied this method to analyze hTERT expression in myelodysplastic syndrome (MDS). Nineteen acute myelogenous leukemia and 40 MDS patients samples were obtained, 36 patients were diagnosed as low risk MDS (RCUD, RCMD, MDS-U), 14 patients were diagnosed as high risk MDS (RAEB) according to WHO classification. All samples were acquired after informed consent was obtained from the patients. Expression of hTERT protein in CD45 dull blast population was analyzed and it was higher in $CD34^+$ than $CD34^-$ cells. The percentage of the $CD34^+$ cells expressing hTERT ranged from 9.66% to 90.91% in low risk MDS patients, whereas from 50.46% to 97.68% in high risk MDS. The expression level was higher in the high risk group compared to that in the low risk group in MDS (P=0.0084). This observation implied that telomerase up-regulation and hTERT expression were important for disease progression and could be a marker of more advanced disease. Then we examined the hTERT expression in $CD34^+$/CD38 high cells and $CD34^+$/CD38 low cells containing stem cell fraction in 26 MDS and 11 AML bone marrow specimens. Of interest, hTERT expression in CD34+/CD38 low cells were higher than in $CD34^+$/CD38 high cells (P=0.021) in MDS but no significant difference was found in AML (P=0.217). $CD34^+$CD38 low cells in high risk MDS demonstrated higher hTERT expression compared to those cells in low risk MDS (P=0.02). This observation is inconsistent with previous reports describing normal bone marrow hematopoietic cell findings. We speculated that this phenomenon could be a marker of MDS abnormality and that telomerase up-regulation may be initiated in the more primitive precursor fraction containing hematopoietic stem cells during the disease progression. Telomerase studies may be useful for definition of the risks associated with disease severity. Multi-parameter nature of flow cytometry and its ability to identify cellular sub-populations will facilitate a fuller understanding of the mechanisms of activation of telomerase.

0903

HETEROGENEOUS EXPRESSION PATTERN OF HEDGEHOG SIGNALLING PATHWAY IN HEMATOPOIESIS OF MYELODYSPLASTIC SYNDROME

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Background. Recent investigations provide evidence that the Hedgehog (HH) pathway is essentially involved in apoptosis, proliferation and differentiation of hematopoietic cells via autocrine or paracrine signalling. *Aims.* Therefore, we investigated the expression pattern of the HH signalling components in hematopoiesis of different stages of myelodysplastic syndromes (MDS) compared to control samples from healthy donors. *Methods.* 18 normal controls and 80 cases with MDS (subgroups according WHO: n=40 refractory cytopenia (RA) with multilineage dysplasia, n=13/22 refractory anaemia with excess of blasts I/II and n=5 secondary acute myeloid leukaemia) were included in the investigations. All MDS cases were fully characterized for dysplastic change of bone marrow as well as for risk factors (IPSS), laboratory parameters and follow-up. Bone marrow sections were analyzed by immunohistochemistry for HH ligands (Desert, Sonic, and Indian HH), receptors (Smoothened (Smo), Patched and HIP) and transcription factors Gli (1-3). Additionally, the findings were related to mRNA expression levels of MDS and control samples. *Results.* Overall, the mRNA and protein levels of HH signalling components were low and increased from ligands to transcription factors in the hematopoiesis. Most significant differences could be evaluated inside granulopoiesis (especially for Smo and Gli, ANOVA P<0.05) comparing controls and cases as well as different IPSS scoring levels. Interestingly, up- or down-regulation of HH signalling members could be detected inside different MDS subgroups. Finally, significant independent predictors of the HH signalling family for survival probability could be evaluated. *Summary.* Our investigation revealed that (i) HH signalling pathway is significantly heterogeneous expressed in normal and dysplastic hematopoiesis, and that (ii) expression of some particular HH signalling components is partly associated with the survival rate of patients with MDS.

0904

CLINICAL AND BIOLOGICAL FEATURES OF MYELODYSPLASTIC SYNDROMES WITH BONE MARROW FIBROSIS

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Background. Owing to the notable clinical heterogeneity of Myelodysplastic Syndromes (MDS), ranging from indolent conditions developing over years to forms rapidly progressing to leukemia, clinical decision-making and the timing of interventions are still a matter of debate. *Aims.* To search for unifying biological and clinical features stratifying MDS patients in distinct subgroups, so as to improve the evaluation of prognosis and the prediction of clinical outcome in MDS patients. In several studies bone marrow histological pictures have emerged as an important prognostic parameter influencing marrow insufficiency and MDS

outcome. *Methods.* We retrospectively collected clinical and hematological data, including bone marrow fibrosis (MF), on 180 consecutive patients with a diagnosis of MDS. All patients were reclassified according to the WHO criteria; 43 patients were excluded because their disease was classified as CMML (23 patients) and AML with more than 20% marrow blasts (20 patients). The grading of MF was established according to the European Consensus guidelines. Cytogenetic analysis was successful in 97 (69%) out of 137 patients; the IPSS and WPSS scores at diagnosis were also assessed in these 97 patients. *Results.* Of the 137 evaluated patients, 43 (32%) showed grade 0 fibrosis, while 94 (67%) showed pictures of marrow fibrosis ranging from mild (grade 1) to severe (grade 3), in 36 (26%), 52 (38%) and 6 (4%)patients, respectively. There were no significant differences between patients without MF and those with grade 1 MF, regarding clinical characteristics, karyotype and outcome, so we unified patients with grade 0 and grade 1 fibrosis in a single subgroup (58% of patients) and matched their clinical findings and outcome with those of the grade 2 and 3 fibrosis subgroups (42%). Among these two populations we found that MF is significantly related to more severe thrombocytopenia, splenomegaly, a poor-risk cytogenetic status, bi and trilineage dysplasia, a higher risk of leukemic evolution and poor O.S. *Conclusion.* Besides the main changes in hematopoiesis and the cytogenetic pattern , that are the well-known, distinctive hallmarks of MDS providing significant prognostic information, it is becoming apparent that other cellular and mesenchymal marrow components not involved in the neoplastic clone have a role in marrow insufficiency and leukemic transformation. Evaluation of marrow histology will thus be an essential tool in predicting prognosis in MDS patients, with the presence of MF as an independent unfavorable prognostic factor. Moreover, the evaluation of MF in the setting of the new therapeutic strategies (antiangiogenetic, epigenetic) will probably provide new insights into their biological activity and impact on the clinical outcome.

0905

COMORBIDITIES DO NOT MODIFY OUTCOME AND FREQUENCY OF ADVERSE EVENTS DURING AZA, BUT INFLUENCE SURVIVAL

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Background. Myelodysplastic syndromes (MDS) are affecting mainly elderly patients, and age is considered per se a negative prognostic factor. Most of the elderly patients have comorbidity that presumably negatively impact the overall survival and quality of life and may influence response to therapy. A subanalysis demonstrated that MDS patients aged >75 yrs treated with azacitidine have a significantly longer overall survival respect to best supportive care treated patients (Fenaux, Lancet Oncol. 2009). *Aims.* We wanted to verify whether elderly and very elderly patients receiving azacitdine in our Center, outside clinical trials, were less prone to respond to azacitidine or presented more side effects related to therapy. We also analyzed number of comorbidities their impact on the response and management of azacitidine treatment. *Methods.* We analyzed 39 elderly MDS patients (IPSS INT-1 14/39 and INT-2/high 27/39) treated with subcutaneous Azacitidine 75 mg/kg/day for 7 days every 28. Mean number of cycles was 11 (range:42-2). Mean age was 70 yrs (60-82); 33% of patients (13/39) were >= 75 yrs and 38% of the latter (5/13) >= 80 yrs. *Results.* Overall response rate according IWG criteria 2006 was 44%, stable disease was obtained in 34.1 % of MDS patients. Response to azacitidine treatment was evaluated according to IWG 2006 criteria as CR, PR, HI, SD, DP. We demonstrated by Fisher test that these IWG responses did not correlate with age. We also evaluated Charlson comorbidity index (26 patients scored 0, 9 patients 1 and 4 patients >= 2) the Cumulative Illness Rating Scale (CIRS) (24 patients scored 0, 12 patients 1 and 4 patients >= 2) and the Adult Comorbidity Evaluation-27 in relation to age and to hematological response and no correlation was showed. Respectively 75% and 64% of patients did not suffer from any hematological or non hematological adverse events. Adverse events were uniformly distributed independently from age. Median overall survival (OS) of our patient cohort was comparable to that obtained in AZA-001 trial (29,5 months *vs.* 24,6). Median OS in patients < 75yrs and >= 75 yrs was not significantly different (p value > 0.7). In conclusion very elderly patients with comorbidities may be treated with success with azacitidine, without any substantial increase in AE. Nevertheless comorbidities negatively influence overall survival.

0906

DETECTION OF MOLECULAR TARGETS ON THE SURFACE OF CD34$^+$CD38$^-$ STEM CELLS IN MDS

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Background. Myelodysplastic syndrome (MDS) is a kind of clonal stem-cell disorder in which aberration within a haematopoietic stem cell (HSC) gives rise to the entire disease as in acute myeloid leukemia (AML). A lot of studies on the phenotype of leukemic stem cell have showed that contrasting normal stem cells, AML stem cells express substantial amounts of CD96, CD123 and the C-type lectin-like molecule-1 (CLL-1), but lack the expressions of CD90, although both normal HSC and leukemic stem cells are resided within CD34$^+$CD38$^-$ population. So far, however, little is known about expression of the markers and targets on MDS HSC. *Aims.* To investigate whether there are some specific cell surface antigens expressed on MDS HSC that could distinguish MDS stem cells from normal/reactive stem cells, and the correlation of abnormal antigen expression profile to development, progression and risk stratification in MDS. *Methods.* We analyzed the immunophenotypic characteristics of CD34$^+$CD38$^-$ bone marrow (BM) cells from MDS patients compared to normal/reactive BM. Expression of a series of target antigens on CD34$^+$CD38$^-$ cells were analyzed by multi-color flow cytometry in 38 patients with MDS and 10 patients with nonneoplastic hematologic diseases as control using a FACS CaliburTM. Positivity was defined as no less than 10% of cell expression on CD34$^+$CD38$^-$ cells, unless CD90 (≥5%). *Results.* In both groups, CD34$^+$CD38$^-$ progenitors co-expressed CD13, CD33, CD117, CD133 and HLA-DR almost in all patients, but in MDS they expressed obviously higher amounts of CD13 (79±16% *vs.* 36±13%, P<0.05) and CD133 (66±20% *vs.* 25±13%, P<0.05). CD90 was found to be expressed in all control patients but just in 63% of MDS patients, and there was significant difference in expression amounts between the two groups (32±19% *vs.* 12±3%, P<0.05). By contrast, no patients in control group had an expression of CD2, CD5, CD7, CD44, CD96 and CD123 on CD34$^+$CD38$^-$ cells, which expressed variable amounts in 17~53% of MDS patients: CD2 (47±22%), CD5 (44±28%), CD7 (20±9%), CD44 (35±15%), CD96 (43±19%) and CD123 (54±23%). The level of CD13 on CD34$^+$CD38$^-$ cells in RCMD (89±7%), RAEB-1 (88±11%) and RAEB-2 (81±13%) groups were obviously higher than that of RA group (63±16%, P<0.05). Lymphoid markers such as CD2, CD5 and CD7 were more frequently observed in RAEB subtype or INT and HIGH-R cases, although the level of these markers was rather low. *Conclusions.* MDS stem cells display complex phenotypic abnormalities different from both normal stem cells and AML stem cells and risk-associated alterations, which may contribute to the evaluation of development, progression and risk-stratification in MDS and be a potential prognostic indicator in the future.

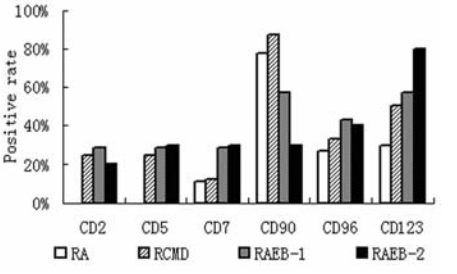

Figure. Antigen expression in MDS subgroups.

0907

IN VITRO STUDY OF BIOLOGICAL CHARACTERISTICS AND FUNCTIONS OF MESENCHYMAL STEM CELLS IN LOW-RISK MYELODYSPLASTIC SYNDROMES

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Background. The myelodysplastic syndromes (MDS) include a diverse

group of clonal and potentially malignant bone marrow disorders. Evidences exist that microenvironment cells from MDS marrow show functional abnormalities, which may be relevant to the incidence of such a disease. Mesenchymal stem cell (MSC) is a very important component of hematopoietic microenvironment. *Aims.* This study focused on the biological characteristics and functions of MSC derived from patients with MDS in low-risk. *Methods.* MSC from Bone marrow samples of 11 low-risk MDS patients were isolated, cultured and expanded. Morphology, immunophenotype and osteoblasts differentiation were analyzed. Their capacity of proliferation and hematopoietic supporting *in vitro* were measured. A real-time quantitative reverse transcriptase polymerase chain reaction method (RQ-RT-PCR) was estiblished for detecting the expression levels of relative cytokines and chemokines in MSC. MSCs from healthy donors were obtained as controls. *Results.* Culture-expanded cells from MDS patients present a typical fibroblast-like morphology. Cells were positive for SH2 (CD105), SH3(CD73), Thy-1(CD90), while negative for CD34 and CD45. After induction, these cells could differentiate into osteoblasts. The proliferative ablity of MSCs in MDS patients are not different from those of MSC isolated from normal bone marrow (P>0.05), however, their capacity of hematopoietic supporting *in vitro* were significantly weaker (P<0.05). SDF-1 gene expression levels in MSCs of low-risk MDS patients were significantly higher than that in MSC derived from healthy donors by RQ-RT-PCR (P<0.01), which were further confirmed by ELISA assays in protein level. *Conclusions.* It is inferred that the abnormal function of MSC may influence the regulation of hemotopoiesis in the bone marrow microenvironment of MDS patients. It is worthy of further investigation of new clue of etiological mechanism and therapeutic stratigies of MDS.

0908

VALPROIC ACID AND AZACITIDINE COMBINATORIAL EFFECT ON PHOSPHOINOSITIDE-PHOSPHOLIPASE C (PI-PLC) BETA1 AND CYCLIN D3 IN HIGH RISK MYELODYSPLASTIC SYNDROMES

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Background. Myelodysplastic syndromes (MDS) are clonal haematopoietic stem cell disorders characterized by ineffective haematopoiesis in one or more of the lineages of the bone marrow. The disease can result in a slow decrease in blood cell counts, but it may also have a more aggressive evolution, that is a worsening severe cytopenia or, in about 30% of all the patients, transformation into acute myeloid leukemia (AML). Azacitidine, a DNA methyltransferases inhibitor, has been proven effectiveness in prolonging survival and delaying evolution into AML, alone or in combination with the histone deacetylase inhibitor valproic acid (VPA), which improves the clinical response induced by azacitidine alone. Nuclear inositides are essential co-factors for several nuclear processes, including DNA repair, transcription regulation and RNA dynamics in normal and pathological conditions. Namely, nuclear phosphoinositide-phospholipase C (PI-PLC) beta1 appears to play a fundamental role as a checkpoint in the G1 phase of the cell cycle, mainly targeting cyclin D3. Moreover, PI-PLCbeta1 has been demonstrated to be a marker for monitoring the effect of azacitidine on high risk MDS patients. *Aims.* The clinical observation that VPA enhances the clinical response given by azacitidine in high risk MDS, as well as the fact that at a biological level azacitidine has been demonstrated to induce the expression of lipid signaling molecules, prompted us to further investigate the effect of these combinatorial strategies on nuclear inositide signalling. *Methods.* The effect of azacitidine and VPA on inositide signaling pathways was studied on MDS (IPSS risk: intermediate-2 or high) receiving the combination of azacitidine (75 mg/sqm/die SC for 7 days/28 days) with VPA (600-1.500 mg/die orally). Their results were compared with patients receiving only best supportive care and a pool of healthy subjects. We also analyzed the effect of azacitidine and VPA, alone or in combination, on HL60 cell line, which shows a hyper-methylation of PI-PLCbeta1 and is affected by azacitidine treatment. We performed MTT experiments, cell cycle analyses and quantified the gene and protein expression of PI-PLCbeta1, cyclin D3 and p-Akt before and after the treatment. *Results.* We report that p-Akt, PI-PLCbeta1 and cyclin D3 are affected by azacitidine and VPA, alone or in combination. In particular, the combination of the two treatments not only increases the demethylation effect of azacitidine on PI-PLCbeta1, but also induces the cell cycle progression in the G1 phase. This is demonstrated by an increase in both PI-PLCbeta1 and cyclin D3 expression, as well as MTT and cell cycle analyses. As for p-Akt, it is slightly down-regulated by azacitidine and by the combination of azacitidine with VPA. *Summary/Conclusions.* Our findings indicate that VPA enhances the effect of azacitidine on inositide signalling pathways. *in vitro* experiments on HL60 cells showed that these treatments increase PI-PLCbeta1 and cyclin D3, thus inducing cells towards the G1 phase of the cell cycle. Taken together, our results demonstrate for the first time that, at a molecular level, azacitidine and VPA can modulate key molecules involved in cell cycle progression, thus hinting at a role for these molecules in the MDS pathogenesis.

0909

DECREASE OF TELOMERE LENGTH IN BONE MARROW HEMATOPOIETIC CELLS IS AN ADVERSE PROGNOSTIC FACTOR IN PATIENTS WITH MYELODYSPLASTIC SYNDROME: MEASUREMENT OF TELOMERE LENGTH OF INTERPHASE NUCLEI BY QUANTITATIVE FLUORESCENCE *IN SITU* HYBRIDIZATION METHOD

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Background. Telomere is a region of repetitive DNA at the end of a chromosome, which is essential for cell division and protects a cell's chromosome from abnormalities. We assessed the length of telomeres in myelodysplastic syndromes (MDS), characterized by paradoxical coexistence of hyperplasia of dysplastic hematopoietic cells and enhanced apoptosis of hematopoietic cells, resulting in peripheral cytopenias. *Aims.* The aim of this study was to examine telomere length and to assess the prognostic significance of telomere length of bone marrow interphase nuclei in patients with myelodysplastic syndromes by quantitative fluorescence *in situ* hybridization (FISH). The relative telomere length was presented by telomere to centromere ratio. *Methods.* Quantitative FISH with peptide nucleic acid probe of centromere and telomere was performed on bone marrow nucleated cells of 54 patients with MDS and 4 healthy donors. The relative telomere length presented by telomere to centromere (T/C) ratio was measured with an automated image analyzer. *Results.* Patients with MDS had shorter telomere length than the normal healthy donors (P=0.007). The T/C ratio had a tendency of decrement with age but with no statistical significance. Higher than average relative telomere length group showed poorer survival (P=0.045). Multivariate analysis showed old age, lower relative telomere length, no history of hematopoietic stem cell transplantation, low International Prognostic Scoring System grade and acute myeloid leukemia transformation as independent poor prognostic factors for survival (P=0.012, 0.034, 0.048, 0.013, 0.027, respectively). *Summary.* The relative telomere length presented as T/C intensity ratio of interphase nuclei can be useful as a prognostic factor in myelodysplastic patients. Advantages of interphase measurements include the automated measurement and the possibility of studying nonproliferating cells without the need of cell culture, thus avoiding selection.

Table 1. Multivariate Cox proportional hazard analysis of 54 patients with myelodysplastic syndrome (overall survival)

Variables	P value	Hazard ratio	95% confidence interval
Age	0.012	1.058	1.013-1.106
International Prognostic Scoring System	0.013		
Hematopoietic stem cell transplantation	0.048	0.09	0.008-0.979
Acute myeloid leukemia transformation	0.027	5.073	1.200-21.439
Telomere/centromere ratio *100	0.034	7.655	1.172-49.984

0910

ANALYSIS OF MICRO RNA EXPRESSION DURING ERYTHROID DIFFERENTIATION OF RARS MYELOID STEM CELLS IN COMPARISON WITH NORMAL BONE MARROW STEM CELLS

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Background. The MDS subtype refractory anemia with ring sideroblasts (RARS) is characterized by profound erythroid apoptosis, at least 15% ring sideroblasts of bone marrow erythroblasts, hyperplastic ineffective erythropoesis, mitochondrial ferritin accumulation, and constitutive cytochrome c release from the mitochondrial intermembrane space. BM progenitors are clonal, as shown by HUMARA analysis. The molecular basis for the abnormal iron accumulation, defect mitochondrial function and ineffective heme biosynthesis in RARS remains unknown. Recently there have been several publications regarding regulation of erythroid differentitation by miRNA expression. In this study we investigated the expression pattern of specific miRNAs in erythroid differentiation cultures from RARS and normal CD34$^+$ cells. *Aims.* The aim was to identify specific miRNAs, which expression patterns are significantly different during RARS and normal erythroid differentiation in order to shed light over the vast differences in gene expression in these two cell populations. *Methods.* Bone Marrow aspiration samples collected from RARS-MDS patients and healthy controls according to national ethical requirements were subject to CD34$^+$ cell isolation by Immuno-separation. Cells were cultured over 14 days to differentiate to mature GPA$^+$ erythroblasts. Samples were collected at 0, 4, 7, 11 and 14 days. Total RNA was extracted and expression of miRNAs was analysed using commercial miRNA assays. *Results.* Based on previously published papers on erythroid differentiation, 9 miRNA was selected (miR-15a, miR-15b, miR24, miR-144, miR-150, miR-155, miR-221, miR-222 and miR-451). Significant differences in miRNA expression between RARS and normal bone marrow was observed for miR24, miR-155 and miR-221. In spite of morphological maturation, several miRNAs were overexpressed in RARS at day 14 indicating a defect in differentiation (miR-24 3 fold, miR-155 4 fold and miR-221 4 fold).*Summary/Conclusion.* We report pronounced differences in miR-24, miR-155 and miR-221 expression during erythroid differentiation of RARS compared to normal progenitors. Functional studies will be performed to validate the role of each of these miRNAs in the acquisition of the RARS phenotype.

0911

SURVIVAL OF PATIENTS WITH MYELODYSPLASTIC SYNDROMES DIAGNOSED IN THE AREA OF SOUTH WESTERN GREECE DURING THE PERIOD 1990-2009

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Background. Myelodysplastic syndromes (MDS) are acquired hematopoietic stem cell disorders, characterized by ineffective hematopoiesis, peripheral blood cytopenias, and an increased risk of leukemic transformation. Overall survival of patients with MDS has been associated with the percentage of marrow blasts, number of cytopenias, cytogenetics, and transfusion rate. The precise impact of comorbidities on survival has not yet been fully elucidated. *Aim.* To examine the impact of disease characteristics and comorbid conditions on the overall survival of patients with MDS in the region of South Western Greece. *Methods.* Data from medical files and electronic databases of all hospitals, diagnosing and treating patients with MDS were retrieved. All patients diagnosed with an MDS in the area of South Western Greece between January 1st, 1990 and December 31st, 2009 were retrospectively analyzed. Mean and median overall survival of patients were estimated according to clinical data such as gender, age at diagnosis, residence in a rural or urban area, MDS subtype according to FAB and WHO classification, karyotype and IPSS score. In addition, the effect of comorbid conditions on survival was also estimated. The analysis was performed by SPSS version 17.0 using Kaplan Meier estimates, and Cox regression model. *Results.* Totally, 771 patients with an MDS have been diagnosed during the above-mentioned period. According to FAB classification 279 patients had RA, 78 RARS, 241 RAEB, 32 RAEB-t, 119 CMML and 22 were unclassified. The mean overall survival for the whole patient population was 38.3 months [CI 95% 34.1-42.5] and the overall median survival time was 21.1 months [CI 95% 18.5-23.7]. Median survival was 37.9 months for RA [CI 95% 29.6-46.2], 34.2 [CI 95% 23.0-45.4] for RARS, 13.3 [CI 95% 11.7-14.9] for RAEB, 11.0 [CI 95% 6.4-15.6] for RAEB-t, and 21.1 [CI 95% 14.8-27.4] for CMML. Analyses with unadjusted Cox proportional hazard models showed that older age at diagnosis had a significant negative impact on survival (P<0.0001, HR=1.83, 95% CI=1.56-2.15), whereas gender and residence in a rural or urban area did not have any impact (P=0.985 and P=0.113 respectively). FAB and WHO subtypes with increased marrow blasts (P<0.0001), Int-2 or High IPSS score (P<0.0001) and poor karyotype according to IPSS (P=0.023) were also associated with shorter survival. Comorbid conditions were consequently taken into consideration in a multivariate Cox regression model, where diseases, such as congestive heart failure (P=0.033, HR=1.43, 95% CI=1.03-1.99) and peptic ulcer (P=0.035, HR=0.77, 95% CI=0.60-0.98) appeared to have an impact on survival, whereas other conditions, such as coronary heart disease, diabetes mellitus and COPD were not shown to have a significant impact on survival. *Conclusive remarks.* The impact of various comorbid conditions on the survival of patients with MDS needs to be further investigated. Specific comorbidity scores taking into consideration the real impact of various diseases on the course of MDS should be created and verified.

0912

TELOMERE SHORTENING AND AML PROGRESSION IN PATIENTS WITH MYELODYSPLASTIC SYNDROME AND DELETION 5Q TREATED WITH LENALIDOMIDE

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Background. Lenalidomide has been shown to induce transfusion independence and cytogenetic response in a high proportion of patients with MDS and isolated 5q deletion (del5q). However, some of these patients progress into acute myeloid leukemia, particularly those without a cytogenetic response, and those who develop complex karyotypes. Yet the mechanisms inducing the leukemic transformation still have to be elucidated. Since dysfunctional telomeres play an important role in the development of genetic instability and since shorter telomeres are associated with advanced MDS and AML, we reasoned whether telomere shortening may contribute to leukemic progression in patients with MDS and del5q. *Aims.* To determine whether telomere length is associated with the risk of leukemic transformation in patients with MDS and del5q treated with lenalidomide. *Methods.* This study included 14 patients with transfusion-dependent anemia and low- or intermediate-1-risk MDS enrolled in the studies MDS-003 (n=42) or MDS-004 (n=260) who were treated with lenalidomide according to the study protocols. Written informed consent was provided according to the Declaration of Helsinki. 7 patients progressed into RAEB-1 or AML while on study and 7 patients did not. Time span between diagnosis and study entry was similar in both groups. Combined fluorescence R-banding and T/C-FISH analysis to determine the telomere length of each individual chromosome was performed as described earlier (Lange *et al.* 2009). *Results.* Telomere length at study entry was 5.9 kb (range 4.8 to 7.9 kb) in patients with MDS and del5q who later underwent leukemic transformation. Patients without later disease progression had a median telomere length of 9.3 kb (range 8.4 to 10.2kb). Thus, patients, who progressed after a median of 25 months (range 5 to 51 months) had significantly (P<0.05) shorter telomeres than patients who had a cytogenetic response or stable disease. Notably, during treatment with lenalidomide, telomeres had a median increase in length of 2.9kb (range: 0.5 to 3.4kb) in the group of patients who underwent leukemic transformation. Three of the patients acquired additional chromosome aberrations and developed complex karyotypes. They had a median telomere length of 6.4kb (range: 4.8 to 7.9kb), which was not significantly different from the median telomere length of 5.8 kb (range: 4.8 to 7.5kb) in the group of patients who showed an isolated del5q during the leukemic transformation. *Summary.* Telomere shortening seems to predispose to leukemic transformation in patients with MDS and del5q. These data need confirmation in larger patient cohorts, before telomere length measurement can be used for risk assessment prior to starting lenalidomide treatment.

Lange K, Holm L, Vang Nielsen K, Hahn A, Hofmann W, Kreipe H, Schlegelberger B, Göhring G. Telomere shortening and chromosomal instability in myelodysplastic syndromes. Genes Chromosomes Cancer. 2010;49:260-269.

0913

LOW FREQUENCY OF ASXL1 MUTATIONS IN A SERIES OF 42 PATIENTS WITH REFRACTORY ANEMIA WITH RING SIDEROBLASTS

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Background. The identification of genes which are frequently mutated in myelodysplastic syndromes (MDS) is critical to a full understanding of the molecular pathogenesis of these disorders. Recently, mutations of the *ASXL1* (additional sex combs 1) gene have been reported in 11% of MDS patients, 43% of chronic myelomonocytic leukemia patients and 25% of acute myeloblastic leukemias. However, preliminary available data suggest that *ASXL1* mutations seem to be a rare event in early (i.e. low-blast) subtypes of MDS. *ASXL1* belongs to a family of three identified members that encode poorly characterized proteins regulating chromatin remodelling. The *ASXL1* gene is located in the chromosomal region 20q11, a region commonly affected in malignancies of the myeloid lineage. Several studies suggest that *ASXL1* may function as a tumor suppressor gene in myeloid malignancies by affecting stem or progenitor cell self-renewal or differentiation. Reported mutations, located in exon 12 of the gene, are mostly frameshift mutations caused by deletion or duplication of a nucleotide and are predicted to lead to the truncation of the C-terminus of the protein, which contains a PHD finger. This could prevent it from binding methylated histone lysines and interacting with chromatin modifiers, thus enhancing self-renewal in hematopoietic progenitors. *Aim.* To screen *ASXL1* mutations in a series of patients with refractory anemia with ring sideroblasts (RARS) in order to determine their frequency and any potential association with disease outcome. *Patients and Methods.* Bone marrow samples obtained at diagnosis from 42 *de novo* RARS (FAB criteria) were studied [27M/15F; median age: 71 yr. (range: 44-87); median Hb level, 9.9 g/dL (5.9-16.9); median platelet count, 271×10^9/L (67-852) and median absolute PMN count, 3.5×10^9/L (5.9-16.9); median marrow blasts, 1% (0 - 4); and ring sideroblasts, 37% (15-78)]. According to IPSS, 40 patients belonged to the low-risk category whereas the rest two patients were intermediate-1. According to WHO 2008 criteria, patients were classified as RARS (n=23), RCMD (n=18) and '5q syndrome' (n=1). Genomic DNA was amplified using specific primers for ASXL1. Direct sequencing was performed on an ABIPRISM 310 DNA Analyzer (Applied Biosystems, Foster City, CA). Sequence analysis was checked by GeneBank Accession NT_011362. *Results.* Among the 42 patients, only a single heterozygous missense mutation c.3147C>T p.Val908Ala of ASXL1 was found (2.3%). In addition, we found three previously reported polymorphisms and five different non-reported synonymous single base changes that could be theoretically polymorphisms. *Conclusions.* Our results confirm that *ASXL1* mutations are an uncommon event in RARS.

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0914

TELOMERE MAINTENANCE IN MYELOID MALIGNANCIES

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Background. Telomeres are regions of highly repetitive GC-rich DNA sequence at the end of eukaryotic chromosomes that act to protect loss of genetic information due to incomplete DNA replication at the ends of chromosomes in late S phase. Telomeres are progressively shortened with cell divisions and trigger cellular senescence in somatic cells. Most stem cells and cancer cells are able to proliferate indefinitely, because of a telomere maintenance mechanism as activation of telomerase, a ribonucleoprotein that synthesizes telomeric DNA sequences and almost universally provides the molecular basis for unlimited proliferative potential. Loss of telomerase function produces short telomeres, potentially resulting in chromosome recombination, end-to-end fusion and recognition as damaged DNA. Regulation of hTERT expression depends on different transcription factors that bind hTERT promoter and positively or negatively control hTERT expression. In particular the c-Myc and Mad1 network can regulate hTERT expression through the binding to E-box binding sites. *Aims.* In this study we evaluated telomerase enzymatic activity (AT), telomeres length and hTERT expression, in mononuclear cells (MNC) from bone marrow at diagnosis of 60 patients with AML *de novo* and secondary to Myelodysplastic Syndrome (AML) and High Risk Myelodysplastic Syndrome (HR-MDS), (n=23) and Low Risk Myelodysplastic Syndrome (LR-MDS, n=37). We also evaluate the expression of c-myc, mad-1 and Wilms' tumor 1 gene (WT1), transcription factors acting over hTERT promoter. These results are compared with the control (normal bone marrow, n=10) to increase our knowledge upon telomere maintenance mechanism and correlation to the clinical features in MDS and AML patients. *Methods.* The telomerase activity (AT) was quantified using a real-time PCR-based telomeric repeats amplification protocol. All gene expressions were determined by real-time PCR. Telomere length was analyzed by Telomere Restriction Fragments (TRFs) length method. *Results.* Compared with the controls, telomerase activity (AT) was increased 2- to 6- fold in 8% of low risk MDS patients and 2- to 6-fold in 13% of HR-MDS and AML patients. HTERT expression is higher than in controls in 40% of LR-MDS and in 50% of HR-MDS and AML patients. Levels are not homogeneous among groups. WT1 levels were not increased in LR-MDS patients, in 70% of HR-MDS and AML patients were significantly higher than in control bone marrow. Mad expression was significantly higher in AML and in all MDS samples than in controls. Preliminary results suggest no significantly differences in controls and patients for c-myc expression. Despite of the increased AT and hTERT expression, both in MDS and AML patients TRFs length was lower than in controls. *Summary.* Our data suggests that in MDS and AML patients AT and hTERT expression is higher than those found in normal donors. In particular AML and HR-MDS patients showed hTERT and AT generally higher than LR-MDS patients. On the other hand, telomere length was similar in patients with MDS and AML. WT1 levels of AML and HR-MDS patients were higher than LR-MDS and control values and mad expression was higher in AML and in MDS.

0915

FLOW CYTOMETRY PREDICTS TREATMENT OUTCOME IN INTERMEDIATE-2 AND HIGH RISK MYELODYSPLASTIC SYNDROME PATIENTS TREATED WITH 5-AZACITIDINE

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New treatment strategies that potentially change the natural course of intermediate (int)-2 and high risk myelodysplastic syndromes (MDS), such as 5-azacitidine, are emerging. Recently, we reported that flow cytometric analysis of bone marrow in low and int-1 risk MDS is instrumental to identify clinically relevant subgroups. (Westers *et al.*, Blood 2010) Moreover, it was reported that a flow cytometric scoring system, based on aberrancies in the (im)mature myelo-monocytic lineage is predictive for worse outcome in MDS. (Wells *et al.*, Blood 2003, van de Loosdrecht *et al.*, Blood 2008). The current study aimed to investigate the role of this flow cytometry-based scoring system to assess and monitor response to treatment in int-2 and high risk MDS patients treated with 5-azacitidine. Bone marrow aspirates were analysed by flow cytometry in 13 MDS patients who were treated with 5-azacitidine. Aspirates were drawn before treatment and after every three cycles of 5-azacitidine. Response to treatment was evaluated using IWG-2006 criteria. (Cheson *et al.*, Blood 2006). The median age was 67 (range 62-78). Distribution over WHO 2001 categories was RCMD-RS n=1, RAEB-2 n=5, AML with 20-30% blasts n=5 and MDS/MPD n=2. International prognostic scoring system (IPSS) categories comprised int-2 n=5 and high n=4. In 4 patients the IPSS score could not be assessed. Follow up was present in 7 patients, including 4 responders, 1 progressive disease (PD) patient and 2 stable disease (SD) patients; 2 patients stopped due to toxicity, 1 patient died of PD. Median follow up was 6 months (range 3-9). Median pretreatment Hb was 6.4 mmol/L, platelets 36×10^9/L and absolute neutrophil count (ANC) 1.0×10^9/L. Responders had a significant increase in Hb (median 7.8 mmol/L, P=0.01), platelets (291.5×10^9/L, P=0.04) and ANC (1.4×10^9/L, P=n.s.). Non responders (PD and SD) had a median Hb of 6.4 mmol/L, platelets 69×10^9/L and ANC 0.6×10^9/L. The median pretreatment flow score was 6 (range 3-8). Interestingly, responders had a significant decrease in flow score from median 5 to median 2 (range 1-3, P=0.006) after 3 months of treatment. No change in flow scores was seen in PD and SD patients after 3 months of treatment (median=6, range 4-7). A sustained decrease in flow score was seen in 3 responders after 6 months of treatment (median 2, range 1-3) parallel to a further

increase in median Hb (8.8 mmol/L), platelets (176×10⁹/L) and ANC (1.7×10⁹/L). Moreover, loss of aberrant marker expression on myeloid progenitors was only detected in patients who responded to 5-azacitidine treatment. In conclusion, our data indicate that flow cytometry identifies high risk MDS patients who may benefit from 5-azacitidine treatment. Whether or not flow cytometry may be instrumental in selection of SD patients who may benefit from prolonged treatment with 5-azacitidine is under investigation.

0916

EFFECT OF ALTERNATIVE DOSING SCHEDULES OF AZACITIDINE ON PHOSPHOINOSITIDE-PHOSPHOLIPASE C (PI-PLC) BETA1 IN HIGH RISK MDS PATIENTS

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Background. Nuclear inositides are key cellular second messengers with well established roles in signal transduction pathways, being involved in cell proliferation and differentiation. In particular, the nuclear metabolism elicited by phosphoinositide-phospholipase C (PI-PLC) beta1 plays an important role in the control of the balance between cell cycle progression and apoptosis. Recent findings indicate that the lipid signalling pathways can become therapeutic targets in myelodysplastic syndromes (MDS), which are a heterogeneous group of hematological malignancies characterized by an increased although variable risk of evolution in acute myeloid leukemia (AML). Our group previously demonstrated that PI-PLCbeta1 is a target for azacitidine, an inhibitor of DNA methyltransferases which has been proven to be effective in prolonging survival and delaying evolution into AML, and that the reduction of PI-PLCbeta1 methylation could predict the clinical response to azacitidine. *Aims.* The currently approved azacitidine regimen is 75 mg/sqm/die, administered subcutaneously (SC) or intravenously (IV) for 7 days every 28 days. Recently, alternative azacitidine dosing regimens, which avoid week-end dosing, have shown to induce therapeutic responses consistent with the currently approved schedule. This clinical observation prompted us to further investigate the effect of alternative dosing schedules of azacitidine on nuclear lipid signalling, in order to clarify the molecular mechanisms leading to the response to demethylating therapies. *Methods.* From September 2004, 20 MDS patients, with a IPSS risk intermediate-2 or high and a median age of 70 (50-84) years, were treated with azacitidine (AZA), following 3 different treatment regimens. Group 1 (8 patients) received the currently approved regimen (AZA 75 mg/sqm/die SC for 7 days/28 days). Group 2 (6 pts.) received the combination of AZA (75 mg/sqm/die SC for 7 days/28 days) with VPA (600-1.500 mg/die orally) and ATRA (30 mg/sqm/die, orally, from cycle n. 5, in case of non-response). Group 3 (6 pts.) received the alternative 5-2-5 AZA regimen: AZA 50 mg/sqm/die SC for 5 days, followed by 2 days without treatment, then 50 mg/sqm/die for 5 days. We quantified the degree of PI-PLCbeta1 methylation and gene expression before and during azacitidine administration. *Results.* Here we show that the amount of PI-PLCbeta1 is linked to azacitidine responsiveness in MDS patients. Following azacitidine treatment, and in correlation with the therapeutic response, but not with the dose schedule, PI-PLCbeta1 expression increased, whereas PI-PLCbeta1 promoter methylation was reduced in responder patients. Moreover, independently from the dose schedule, we observed that the decrease of PI-PLCbeta1 promoter gene methylation may anticipate the hematologic response, given that the variations in PI-PLCbeta1 gene expression may occur some cycles prior to the hematologic improvement. *Summary/Conclusions.* PI-PLCbeta1, which has been demonstrated to be hyper-methylated in MDS patients and is therefore useful for monitoring the effect of demethylating therapies, can be used for assessing azacitidine responsiveness in high risk MDS independently from the dose schedule.

0917

FLUORESCENT *IN SITU* HYBRIDIZATION (FISH) TECHNIQUE IS A GOOD TOOL FOR IMPROVING DIAGNOSIS OF PRIMARY MYELODYPLASTIC SYNDROMES (MDS)

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Myelodysplastic syndromes are a heterogeneous group of clonal haematological diseases characterized by bone marrow failure with abnormal differentiation of myeloid cells, peripheral cytopenias, dysplastic features in one or more myeloid lineages and enhanced risk of transforming in acute myeloblastic leukaemia (AML). Several efforts have been performed to classify these syndromes evaluating the risk of leukemic transformation (WHO classification, IPSS score). For such evaluation, cytogenetic abnormalities are the most relevant parameter to consider in combination with cytopenias , dysplasia and numbers of bone marrow blast cells. Furthermore, the recent introduction of targeted-based drugs for treating subgroups of MDS, led to an updated of response criteria, including the complete cytogenetic response as for patients treated with Lenalidomide in the 5 q- syndrome. The aim of this work was to evaluate the contribute of fluorescent *in situ* hybridization (FISH) to improve the cytogenetic analysis of MDS to better characterization of the patients. We studied 121 patients suspected to be affected by primary MDS. The conventional cytogenetic analysis was performed on bone marrow samples. 90 patients (74%) showed an informative karyotype (at least 20 metaphases); 25 out of 90 (28%) had a normal karyotype, while 65 (72%) showed chromosomal abnormalities. The 31 cases with none or insufficient number of metaphases, were studied with FISH analysis, using the following probes: 1) LSI CSF1R/D5S23, D5S721 Dual Color Probe for 5q31-35 region, 2) LSI D7S486/CEP 7 Dual Color Probe for chromosome 7 and 3) probe specific for the alpha satellite (centromeric) region, 8p11.1-q11.1. of chromosome 8 (VYSIS). 9/31 cases had an abnormal karyotype (del 5q: 4 cases; +8: 2 cases; - 7: 1 case; del 5q and + 8: 2 cases). FISH technique was consistent with the results of conventional cytogenetic analysis in both positive and negative control. Using conventional cytogenetics and/or FISH analysis, 100% of patients had cytogenetic informations with 61% (74 pts) exhibiting an abnormal karyotype. According to WHO classification (2001), our patients were stratified as: 42 AR, 5 ARS, 42 RCMD, 18 5q- syndromes, 11 AREB and 3 LAM. Using IPSS score the patients resulted in: 37% LOW, 50% Int 1, 6% Int 2 and 7% HIGH risk class. All the eighteen patients affected by 5q- syndrome were treated with Lenalidomide with fairly good results. FISH analysis combined to conventional cytogenetic is able to better classified MDS especially for differential diagnosis between low risk group *vs.* idiopathic cytopenias of uncertain significance (ICUS). Moreover, the identification of defined cytogenetic sub groups (e.g. 5q-) is extremely relevant for using target-based drugs. Finally the identification of cytogenetic marker is the best tool for monitoring response to therapy and/or clone evolution/transformation.

0918

BONE MARROW CELLS FROM MYELODYSPLASTIC SYNDROMES SHOW ALTERED IMMUNOPHENOTYPIC PROFILES WHICH MAY CONTRIBUTE TO THE DIAGNOSIS AND PROGNOSTIC STRATIFICATION OF THE DISEASE: A PILOT STUDY

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Background. A heterogeneous spectrum of immunophenotypic abnormalities have been reported in myelodysplastic syndromes (MDS). However, most studies are restricted to the analysis of $CD34^+$ cells and/or other major subsets of $CD34^-$ cells frequently not exploring the diagnostic and prognostic impact of immunophenotyping. *Methods.* We propose for the first time an immunophenotypic score (IS) based on the altered distribution and immunophenotypic features of maturing/mature compartments of bone marrow (BM) hematopoietic cells in 56 MDS patients that could contribute to a refined diagnosis and prognostic evaluation of the disease. *Results.* Although MDS-associated phenotypes were detected in reactive BM, the overall immunophenotypic profile of BM cells allowed an efficient discrimination between MDS and both normal and reactive BM, once the number and degree of severity of the abnormalities detected per patient were simultaneously considered in the proposed IS. Interestingly, increasingly higher IS were found among MDS patients showing adverse prognostic factors as well as in low- *vs.* high-grade cases. The most informative prognostic factors included: the number of $CD34^+$ cells, presence of aberrant $CD34^-/CD117^+$ precur-

sors, decreased mature neutrophils and CD34$^-$ erythroid precursors, and increased numbers of CD36$^-$/lo erythroid precursors; additionally, the IS was an independent prognostic factor for overall survival. *Summary/Conclusions.* Assessment of immunophenotypic abnormalities of maturing/mature BM cells allows an efficient discrimination between MDS and both normal and reactive BM, once the number and degree of severity of the abnormalities detected are simultaneously scored. Interestingly, progressively higher IS were found among MDS patients with adverse prognostic features and shorter overall survival.

0919

BONE MARROW CYTOKINE CHANGES DURING TREATMENT WITH LENALIDOMIDE IN LOW AND INTERMEDIATE-1 RISK MYELODYSPLASTIC SYNDROMES WITH DEL(5Q)

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Background. Immunological responses are important in the initiation and progression of myelodysplastic syndromes (MDS). It has been suggested that T cells be involved in the pathogenesis of MDS. Cytokine levels, such as IL-7 and IFN-gamma, are associated with low-risk disease. Patients with MDS with del(5q) are generally at low risk with severe anemia requiring transfusions. Treatment with lenalidomide has proven efficacy in MDS patients with del(5q) inducing erythroid responses and suppression of the del(5q) clone; its anti-angiogenic, antiproliferative, and pro-erythropoietic mechanisms remain unclear. It has been shown that lenalidomide inhibits the proliferation and function of T regulatory cells (Tregs). *Aims.* In a multicenter Italian phase II trial to evaluate safety and efficacy of lenalidomide in primary MDS patients with del(5q) and low or Int-1 risk IPSS, we investigated changes in the transcription of cytokines and their receptors during treatment. *Methods.* The starting dose of lenalidomide was 10 mg p.o once daily on a continuous daily schedule for a maximum of 12 months. Bone marrow aspirates were obtained on study entry and every 12 weeks. Assays were performed using TaqMan® Low Density Array Fluidic card (TaqMan® Human Array, Applied Biosystems, Foster City, CA, USA) based on Applied Biosystems PRISM® 7900HT comparative ddCT method, according to the manufacturer's instructions. Target gene expression levels were measured in triplicate and normalized against the expression of the 18S housekeeping gene from a bone marrow pool of normal, healthy subjects at all timepoints. Median relative gene expression values in MDS patients were compared to healthy subjects, set as a value of 1. *Results.* Informed consent was obtained in all patients. Twenty-one patients were evaluated at baseline and after 12 weeks. Mean age was 73±8 years. Mean Hb was 8.7±0.9 g/dL and 17 patients were transfusion-dependent. Five patients had additional cytogenetic abnormalities. Eighteen patients experienced erythroid responses. Significant variations in gene expression of cytokines and receptors were observed during treatment. Genes significantly regulated during lenalidomide treatment ($P<0.05$) are shown in the Table. FAS and IL-7 gene expression were prevalently under-expressed and significantly increased after 12 weeks. Accordingly, IL7R was over-expressed in all patients at baseline and its expression was significantly reduced during treatment. Furthermore, IFN-gamma expression increased during therapy. Summary. The protein encoded by FAS gene is a member of the TNF-receptor superfamily and its interaction with its ligand leads to apoptosis. Interleukin (IL)-7 is an essential cytokine that promotes the proliferation and survival of B- and T-lymphocyte progenitors. The IL7R gene on chromosome 5 (5p13) codifies for the IL7 receptor, which blocks apoptosis during differentiation and activation of T lymphocytes. It functions, in part, through the induction of the expression of the antiapoptotic protein Bcl-2 . The results of the present study indicate that lenalidomide may act through immunological changes. Further detailed analyses in these patients may provide new insights into the pathogenesis of MDS with del(5q), and the long-term effects of lenalidomide treatment on immunological changes in bone marrow cells.

Table 1. Changes in cytokine expression during treatment.

Gene	Baseline median	IQ range	After 3 mos, median	IQ range	*P*-value
FAS	0.03	0.01 – 0.38	4.94	0.66–1382.50	<0.0001
IL-7R	2719.00	1907–4987	0.22	0.03–6.20	0.003
IL-7	0.16	0.05–0.49	12.37	0.16–25.07	0.012
IFN-γ	0.69	0.23 -10.38	45,23	21.41 – 35134.14	0.001

0920

INCREASED GLOBAL DNA METHYLATION AND REDUCED HISTONE H3 (LYSINE 9) ACETYLATION IN PATIENTS WITH MYELODYSPLASTIC SYNDROME

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Background. Epigenetic aberrations are now well recognized as very frequent and also as early events in the process of malignant transformation. These molecular mechanisms regulate gene expression without changing the DNA sequence and include alterations in the methylation status of DNA, covalent modification of histone tails, chromatin remodeling, and microRNAs. Somewhat contradictory, in patients with myelodysplastic syndrome (MDS), the induction of hypomethylation using DNA methyltransferase inhibitors leads to very promising clinical response rates and haematological improvement. *Aims.* We investigated global DNA methylation and histone H3 lysine 9 acetylation by immunohistochemistry in bone marrow trephine biopsy specimens in a cohort of 110 MDS patients comprising all subgroups. Results were compared to an age-matched control group of healthy subjects and to a group of AML patients. *Methods.* Immunohistochemistry was performed on paraffin-embedded sections, using anti-5-methylcytosine/5mc and anti-Acetyl-Histone H3 (Lys9)/AcH3K9 antibodies. Scoring of immunohistochemistry was evaluated with a four-point scale for both the number of positive tumor cells and their intensity of immunoreactivity. *Results.* Our results showed that in MDS the 5mc immunostaining score was intermediate between normal controls and AML cases, whereas the AcH3K9 immunostaining score was lower than normal control. The 5mc score correlated significantly with the risk score according to the International Prognostic Scoring System, the blast count and the karyotype. Our results suggest that global hypermethylation and histone hypoacetylation correlate with MDS aggressiveness. *Summary.* These results may provide a molecular explanation for the success in treating MDS patients with hypomethylation-inducing agents and why patients with a poor karyotype respond best. Future studies have to analyse whether the determination of global methylation and histone acetylation levels may serve as a new predictive marker for therapy response.

Myeloma and other monoclonal gammopathies - Biology 3

0921

HIGH SERUM SCLEROSTIN CORRELATES WITH ADVANCED MULTIPLE MYELOMA AND EXTENDED LYTIC BONE DISEASE; REDUCTION POST BORTEZOMIB MONOTHERAPY

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Background. Osteoblast dysfunction characterizes bone metabolism in multiple myeloma (MM). To-date dickkopf-1 (Dkk-1) is considered as the main osteoblast inhibitor which is overproduced by myeloma cells and inhibits Wnt signaling. Sclerostin is another canonical Wnt antagonist through its binding to LRP 5/6. *Aim.* To evaluate the serum levels of sclerostin in MM patients and explore possible correlations with clinical and laboratory data. *Patients/Methods.* We studied 157 patients (87M/70F, median age 68 years) with MM at diagnosis before the administration of any type of therapy, including bisphosphonates and 25 patients (14M/11F, median age 68 years) with relapsed disease who were treated with bortezomib monotherapy (with the addition of dexamethasone if <PR was achieved after 4 cycles). Serum sclerostin and osteonectin (SPARC) were measured using an ELISA methodology developed by BDF for Biomedica Medizinprodukte (Vienna, Austria). Bone remodeling was studied by the measurement of a series of serum indices within one week from diagnosis in newly-diagnosed patients and on day 1 of cycle 1 and on day 21 of cycles 4 and 8 of bortezomib therapy in relapsed patients: i) osteoclast regulators [sRANKL and osteoprotegerin (OPG)], ii) Dkk-1, iii) bone resorption markers (CTX and TRACP-5b), and iv) bone formation markers [bone-specific alkaline phosphatase (bALP) and osteocalcin]. The above molecules were also determined in 21 MGUS patients and 21 healthy controls, of similar gender and age. *Results.* MM patients at diagnosis had increased levels of serum sclerostin compared with MGUS patients (mean value±SD: 0.48±0.46 *vs.* 0.26±0.29 ng/mL; P=0.004) and healthy controls (0.31±0.20 ng/mL, P=0.01). In contrast, both MM and MGUS patients had reduced levels of serum SPARC (2632±1621 and 2724±1802 ng/mL, respectively) compared to controls (5281±5020 ng/mL; P<0.001). Sclerostin values strongly correlated with beta2-microglobulin (r=0.354, P<0.0001), cystatin-C (r=0.389, P<0.0001), creatinine (r=0.380, P<0.0001), bALP (r=-0.541, P<0.0001) and Dkk-1 (r=0.328, P<0.001). Patients (both newly-diagnosed and relapsed) with advanced bone disease (>3 lytic lesions and/or a pathological fracture) had increased sclerostin values compared to all others (median: 0.58 *vs.* 0.41 ng/mL, P=0.03). Patients with ISS-3 disease had increased levels of sclerostin compared to ISS-1 and ISS-2 patients (ANOVA P=0.001). Median survival of MM patients at diagnosis was 48 months and median follow-up period was 20 months. Patients who had a serum sclerostin of ≥0.62 ng/mL (upper quartile, n=40 patients) had a median survival of 27 months, while median survival of all other patients was 98 months (P=0.031). In relapsed patients, sclerostin levels were increased even compared to newly-diagnosed patients (0.62±0.34 ng/mL; P=0.01). Bortezomib produced a dramatic reduction of sclerostin after 4 cycles of therapy (0.32±0.24 ng/mL; P<0.01) in both responders (n=16) and non-responders (n=9). *Summary/Conclusions.* Our study provides evidence that sclerostin is increased in the serum of MM patients and correlates with advanced disease features. Sclerostin seems to participate in the MM biology and thus it may be a possible target for the development of novel therapies that can both increase osteoblast function and target myeloma cells. Reduction of sclerostin post-bortezomib monotherapy may reflect another mechanism for the restoration of osteoblast function by bortezomib in MM.

0922

SERUM FREE LIGHT CHAINS RATIO: AN INDEPENDENT PROGNOSTIC FACTOR FOR OVERALL SURVIVAL AND PROGRESSION FREE SURVIVAL IN MULTIPLE MYELOMA

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Background. The serum free light chain (sFLC) analysis has been used for the diagnosis and monitoring of plasma cell dyscrasias, also in monoclonal gammopathy of undetermined significance (MGUS) evolving to multiple myeloma (MM). Some studies have evaluated the prognostic value of the sFLC levels expressed as K/L ratio (sFLCR), but this factor has not been introduced yet into the international staging system (ISS) for MM and its associated prognostic factors. *Aim.* We have evaluated in this study, the impact of sFLCR, measured at diagnosis in MM patients, on the progression free survival (PFS) and overall survival (OS). *Methods.* A total of 118 consecutive MM patients diagnosed in our hospital between years 2002 and 2008, and for which we have assessed the sFLCR at diagnosis, were included in this study. There were 73 (62%) males and 45 (38%) females with a median age of 57 years [34-72], 63 IgG (49K & 14L), 35 IgA (18K & 17L), 2IgD (1K & 1L) and 18 light chains (9K &9L). According to the ISS, there were 11 (9%) in stage I, 13 (11%) in stage II and 94 (80%) in stage III. Among 55 (47%) FISH analysis done, 28 (24%) detected a chromosome 13 deletion. Measurements of sFLC were made using the Freelite® test from the Binding site on a BNII®, Dade Behring©. According to the distribution of the different ratios, we have defined three groups: group1 (n=25): patients with 0.13<sFLCR<3.3 which represents the double of the normal range (0.26-1.65); group2 (n=63): patients with sFLCR>3.3 and group3 (n=30): patients with sFLCR<0.13. Kaplan Meier and cox regression analysis were performed to study the PFS and OS in different groups using R statistical software (version 2.9.2) *Results.* After a median follow-up of 38 months [3.3-93.7], the probability of OS at 5 years for groups 1, 2 and 3 was 75% [56-100], 60% [47-76] and 40% [23-69] respectively; and the probability of PFS at 5 years was 69% [49-96], 43% [31-60] and 29% [15-54] respectively (Figure).The multivariate analysis studying age, ISS, sex, cytogenetics and sFLCR, showed that both OS and PFS are worslty affected with a more abnormal sFLCR, Group2 *vs.* Group1: for OS [HR=2.06 (0.7 - 6.06) P=0.19], for PFS [HR=2.63 (1.01 - 6.83) P=0.048]; Group3 *vs.* Group1: for OS [HR=3.14 (0.97 - 10.21) P=0.056], for PFS [HR=3.4 (1.21 - 9.51) P=0.02]. *Conclusion.* Our study has showed that abnormal sFLCR at diagnosis affects OS and more strongly the PFS independently of any other concomitant variable. We suggest that this factor deserves more focus for its validation as a prognostic factor in MM.

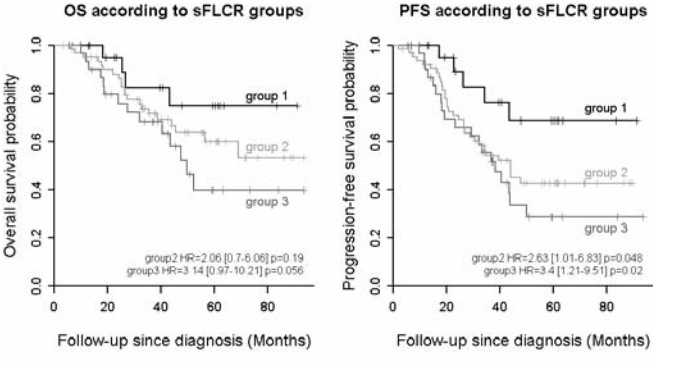

Figure.

0923

STUDY OF CIRCULATING ENDOTHELIAL CELLS IN MULTIPLE MYELOMA: BIOLOGICAL ASPECTS AND EFFECT OF NOVEL AGENTS

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Background. There is accumulating evidence from preclinical studies that circulating endothelial cells (CEC) and their precursors (CEP) (ie. mobilized EC from the BM in response to angiogenic cytokines) are involved in tumor angiogenesis. Rare in healthy individuals, CEC and CEP are increased in cancer patients (pts). CEC or CEP enumeration has been proposed as surrogate biomarker for (anti-angiogenic) treatment response and is currently being applied in multiple clinical studies. Preclinical studies also suggest a role of CEP in tumor regrowth after therapy. Cytotoxic agents administered at MTD induce a rapid CEP mobilization during therapy-free intervals, with possible paradoxal rebound angiogenesis. This CEP mobilization can be inhibited by anti-angiogenic agents, resulting into sustained EC suppression. In MM, angiogenesis is increased in the BM and the effect of novel agents such as Bortezomib (VEL) and Thalidomide (THAL) is partially mediated by inhibition of BM angiogenesis. *Aims.* In the present study, we evaluated CEC and CEP numbers in MM pts and monitored their kinetics during therapy with Melphalan (MEL)-, THAL- and VEL- based regimens. *Methods.* CEC ($CD31^+CD45^-CD34^+CD133^-$) and CEP ($CD34^+CD133^+CD45$dim cells) were enumerated among blood mononuclear cells by multicolor FACS at baseline and at multiple time points during treatment. Baseline levels and changes during treatment were correlated with response and survival. *Results.* At baseline, MM pts (n=71) show significant higher CEC and CEP levels *vs.* healthy controls (n=10) (7 and 2,5-fold increase, P<0,02). CEC numbers where are higher in pts with active *vs.* inactive disease (1,9 fold increase, P<0,05) and in pts with relapsed/refractory disease (2,1 fold increase, P<0,05), reflecting increased angiogenesis associated with disease progression. Therapy with VEL and THAL resulted in a rapid and sustained decline in CEC/CEP numbers. Therapy with MEL initially decreased CEP levels, followed by a transient, significant increase at the end of each cycle. Interestingly, CEP increase was less pronounced by MEL in combination with VEL or THAL, suggesting that VEL/THAL inhibited MEL-induced CEP mobilization. This finding may reflect an additional mechanism for the anti-angiogenic and chemosensitizing effect of VEL and THAL, resulting into increased anti-tumoral efficacy and clinical benefit as shown in recent randomized trials. *Conclusion.* This study shows that CEC and CEP levels are increased in MM pts and correlate with disease progression. In addition, CEC and CEP enumeration may represent a surrogate biomarker to assess prognosis or therapy response in MM. Future large-scale studies are necessary to determine their prognostic and predictive value.

0924

CC-4047 (POMALIDOMIDE) INHIBITS MULTIPLE MYELOMA-INDUCED OSTEOCLAST FORMATION

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Multiple myeloma (MM) is a plasma cell malignancy characterized by high incidence of osteolytic bone lesions due to an increase of osteoclast formation and activation that occur into the bone marrow (BM). The alteration of the RANKL/OPG ratio in BM stromal cells (BMSC) and osteoprogenitor cells in favor of the critical osteoclastogenic factor RANKL is induced by MM cells through the cell contact and it is mainly involved in MM-induced osteoclast formation. MM cells also up-regulate RANKL expression and secretion by activated T cells that contribute to the high RANKL level observed in the MM BM microenvironment. Soluble factors such as CCL3/MIP-1α, IL-3 and IL-7 produced by MM cells contribute to the increase of osteoclast formation both directly and indirectly trough RANKL stimulation. Recent data suggest that thalidomide and its derivative immunomodulatory drug (IMiD) lenalidomide may inhibit osteoclast formation directly through the block of osteoclast maturation. In this study we have investigate the potential *in vitro* effect of the new more potent IMID CC-4047 (pomalidomide) on MM-induced osteoclast formation. First we confirmed that pomalidomide as lenalidomide reduced osteoclast formation from $CD14^+$ progenitor cells in presence of RANKL and M-CSF. Secondly we found that pomalidomide significantly inhibits RANKL expression and secretion by primary BMSC and osteoprogenitor cells obtained from MM patients but not from activated T lymphocytes at concentration ranging from 2 to 100 μM. In addition pomalidomide blunted RANKL up-regulation in BMSC/osteoprogenitor cells induced by MM cells in a cell-to-cell contact co-culture system decreasing the RANKL/OPG ratio level. Consistently the pro-osteoclastogenic property of the conditioned medium of MM cells co-cultured with BMSC/osteporgenitor cells was reduced in the presence of pomalidomide. To go further inside to the capacity of pomalidomide to blunt MM-induced RANKL/OPG imbalance in co-culture, we investigated the effect of this IMID on cell adhesion molecules. We show that pomalidomide significantly reduced the expression of CD49d (VLA-4), a molecule critically involved in RANKL up-regulation, by MM cells co-cultured with or without BMSC/osteoprogenitor cells. On the other hand, we did not find a significant inhibitory effect of pomalidomide on the production of soluble pro-osteoclastogenic factors as CCL3/MIP-1α, IL-3 and IL-7 by MM cells. In conclusion our data suggest that pomalidamide inhibits MM-osteoclast formation targeting the microenvironment through the block of RANKL overexpression induced by MM cells.

0925

CYP2C8 GENE POLYMORPHISM AND BISPHOSPHONATE-RELATED OSTEONECROSIS OF THE JAW IN PATIENTS WITH MULTIPLE MYELOMA

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Background. Bisphosphonates (BP) are considered the standard of care for the prevention of skeletal complications in patients with multiple myeloma (MM). Osteonecrosis of the jaw (ONJ) is an uncommon but potentially serious complication of BP, which is characterized by the presence of exposed bone in the mouth. Dental problems or interventions are risks factors for the development of ONJ. Besides, the risk for ONJ increases with BP treatment duration and has been shown to be 5% - 15% at 4 years. More recently, it has been suggested that the presence of one or two minor alleles of the cytochrome P450, subfamily 2C polypeptide 8 gene (CYP2C8) SNP rs1934951 constitutes an independent prognostic marker associated with the development of ONJ in patients with MM receiving BP therapy. *Aim.* To validate the impact of rs1934951 polymorphism in the development of ONJ in 65 patients with MM treated with zoledronic acid. *Patients and methods.* Sixty-five consecutive patients diagnosed with MM between 1993 and 2008 and receiving treatment with zoledronic acid form the basis of this study. Twenty out of the 65 patients had ONJ and the remaining 45 did not develop ONJ. Using dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP) we compiled all functional information and frequencies data in the polymorphic range in caucasian population from rs1934951. Finally, we analyzed 45 samples from heath volunteers to know the polymorphic distribution in the general Spanish population. DNA samples were extracted from peripheral blood or serum. The rs1934951 polymorphism was analysed by a TaqMan® assay method (TaqMan® SNP genotyping Assay, Applied Biosystems) and was read on a LightCycler 480 Endpoint Genotyping Software.

Table. Allelic distribution of the SNP rs1934951.

		CYP2C8 rs1934951 polymorphism, N (%)				
Study group	N	CC	CT	TT	C	T
Patients with MM	65	54 (83)	11 (17)	0 (0)	119 (92)	11 (8)
With ONJ	20	14 (68)	6 (32)	0 (0)	34 (85)	6 (15)
Without ONJ	45	40 (91)	5 (9)	0 (0)	85 (94)	5 (6)
Healthy volunteers	45	39 (86)	6 (13)	0 (0)	84 (93)	6 (7)
Applied Biosystems' SNP Browser	120	56 (67)	23 (28)	4 (5)	135 (80)	31 (19)

Results. There were 30 (46 %) males and 35 (54 %) females and the median age was 62 years (range 40 to 87). The median follow-up was 18 and 30 months for patients who developed and who did not develop ONJ, respectively. Clinical and biologic characteristics of the patients at diagnosis and their response to therapy were homogeneous between controls and cases. Six (32%) out of the 20 patients who developed ONJ were heterozygous (CYP2C8CT) for SNP rs1934951 in intron 8 of

CYP2Y8 *vs.* 5 (9%) out of 45 patients without ONJ and 6 (13%) of the 45 healthy volunteers (P=0.056). None of the individuals analyzed was found to be homozygous for SNP rs1934951 (CYP2C8TT, T will be denoted as a minor allele). No differences in the homozygous genotype for mayor allele (CYP2C8CC) were observed within the three groups (see Table), the most frequent allele in the three populations studied: 14 (70%) patients in MM with ONJ, 40 (89%) patients in MM without ONJ, and 39 (86%) individuals healthy volunteers. These proportions results were similar to those reported into SNP databases (67%). *Conclusion.* In this independent series, we found a trend towards a higher risk of development of ONJ in those patients with MM with the rs1934951 polymorphism on CYP2C8 gene.

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0926

DICKKOPF-1 IS ELEVATED IN NEWLY-DIAGNOSED, SYMPTOMATIC PATIENTS AND IN RELAPSED PATIENTS WITH MULTIPLE MYELOMA. CORRELATIONS WITH ADVANCED DISEASE FEATURES: A SINGLE-CENTER EXPERIENCE IN 284 PATIENTS

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Background. Dickkopf-1 (Dkk-1) protein is an inhibitor of Wnt signaling and is implicated in the pathogenesis of myeloma bone disease. It disrupts osteoblast differentiation and function and is considered as the main factor responsible for osteoblast exhaustion in multiple myeloma (MM). Although serum Dkk-1 correlates with the presence of lytic lesions at diagnosis, there is very limited data for the Dkk-1 concentrations in different phases of MM. *Aims.* The aim of the study was to evaluate circulating serum Dkk-1 levels in MM patients at different phases of the disease and examine possible correlations with clinical and laboratory data. *Patients/Methods.* We studied 284 MM patients (153M/131F, median age 66 years) at different phases of their disease. Twenty patients had asymptomatic myeloma at diagnosis, 147 symptomatic MM at diagnosis, 29 patients were at the plateau phase of MM and 88 patients had relapsed MM after previous response to therapy. For newly-diagnosed and relapsed patients, serum was stored at the time of diagnosis or relapse, respectively, while for patients at the plateau phase of the disease serum was collected at the time of confirmation of the plateau (at least 6 months with stable M-protein without criteria confirming progression). Serum Dkk-1 was measured using ELISA methodology (R&D Systems, Minneapolis, MN, USA). Evidence of bone involvement was documented using plain radiography in patients at diagnosis or relapse. Dkk-1 was also measured in 20 gender- and age- matched controls. *Results.* The serum Dkk-1 concentrations of symptomatic MM patients at diagnosis (median: 1383 pg/mL, range: 274-32,862 pg/mL) were increased compared to controls (1069 pg/mL, 540-2,709 pg/mL; P<0.001), to asymptomatic patients at diagnosis (1044 pg/mL, 480-2,335 pg/mL; P<0.001) and to patients at plateau phase (1013 pg/mL, 414-1729 pg/mL; P<0.001) (Figure).

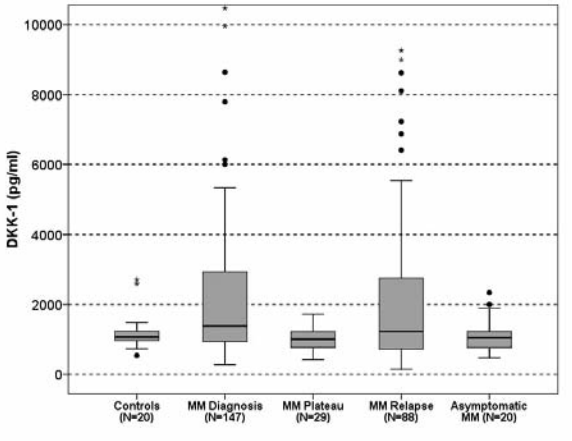

Figure.

There was no significant difference between asymptomatic patients, patients at plateau phase and controls, while relapsed patients had increased Dkk-1 levels (1218 pg/mL, 161-19,325 pg/mL) compared to controls (P=0.001), to asymptomatic patients at diagnosis (P<0.001), and to patients at plateau phase (P<0.001). Patients with ISS-3 myeloma at diagnosis had higher values of DKK-1 than ISS-1 and ISS-2 patients [median Dkk-1 values for ISS-1, ISS-2 and ISS-3 were: 1059, 1290 and 2649 pg/mL, respectively; p(ANOVA)=0.031]. Patients with lytic disease at diagnosis (n=116) had increased levels of Dkk-1 compared with patients with no lytic disease (n=51): 1475 pg/mL, 327-32,862 pg/mL *vs.* 840 pg/mL, 274-1112 pg/mL; P=0.002. Similar results were obtained for relapsed patients (1293 pg/mL, 282-19,325 pg/mL *vs.* 772 pg/mL, 161-2562 pg/mL; for patients with osteolysis (n=77) and for those without osteolysis (n=11), respectively; P=0.01). Dkk-1 serum levels at diagnosis correlated with beta2-microglobulin (r=0.238, P<0.01), LDH (r=0.2, P<0.01) and sRANKL levels (r=0.19, P=0.02), while serum Dkk-1 correlated with serum Ca in all patients (r=0.246, P<0.01). *Conclusions.* Serum DKK-1 is increased in symptomatic MM at diagnosis and in relapsed MM, while serum Dkk-1 of asymptomatic patients at diagnosis and at plateau did not differ from control values. Serum Dkk-1 correlated with advanced disease features, including the presence of lytic lesions. Thus, targeting Dkk-1 may be a treatment option for MM.

0927

SOLUBLE DECOY RECEPTOR 3 (DCR3) MODULATES THE SURVIVAL OF OSTEOCLASTS DERIVED FROM PATIENTS WITH MULTIPLE MYELOMA LYTIC BONE DISEASE

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Background. Decoy receptor 3 (DcR3), belonging to the tumor necrosis factor (TNF) receptor superfamily, is overexpressed in several types of cancer cells and involved in the protection of these cells against the cytotoxic and regulatory effects of apoptotic molecule FasL. The role of DcR3 in the regulation of Multiple Myeloma (MM) osteoclastogenesis has not been investigated so far, although it is unknown why elevated levels of FasL fail to induce osteoclast (OC) apoptosis in MM lytic bone disease. *Aims.* In this study, we purposed to assess the expression of DcR3 in MM lytic bone disease, focusing on DcR3 and FasL interaction, possibly affecting MM OC survival. *Methods.* OCs were obtained from unfractionated PBMCs, freshly isolated from 32 untreated patients diagnosed as having MM lytic bone disease and from 32 control-subjects with monoclonal gammopathy of uncertain significance (MGUS), as previously described. Approval from a local board and patient's informed consent according to the Declaration of Helsinki were obtained. By means of real-time PCR and western blot analysis, we evaluated the expression of DcR3 by T-cells, $CD138^+$ plasma-cells, and human myeloma cell line Karpas 909. DcR3 and FasL were co-immunoprecipitated by anti-FasL MAb in T-cell lysates and OC culture media. OCs from MM lytic bone disease, treated for 48 hr with anti-DcR3 MAbs (R&D System Inc.), were subjected to MTT assay for cell viability, DAPI staining, DNA fragmentation, and lysed and analyzed by western blot to detect the protein levels of caspase-8 and -3. *Results.* We found that T-cells and $CD138^+$ plasma-cells from patients with MM lytic bone disease and Karpas 909 expressed higher levels of DcR3 than T-cells and $CD138^+$ plasma-cells from control-subjects with MGUS. The formation of a DcR3-FasL immunocomplex was detected in both T-cell lysates and culture media of the OCs derived from MM lytic bone disease. In the latter cells, DcR3 neutralization induced the reduction of cell viability, increase of apoptotic cell number, DNA fragmentation, and caspase-8 and -3 activation. *Summary/Conclusions.* In our osteoclastogenesis culture system derived from PBMCs obtained from patients with MM lytic bone disease, OCs displayed a prolonged lifespan in spite of the high levels of apoptotic molecules such as TRAIL and FasL. In the present study, we focused on a possible binding and inactivation of FasL by any molecule present in our culture system. Our findings showed the overexpression of DcR3 by freshly prepared *ex vivo* T-cells and plasma-cells from the patients with MM lytic bone disease, and they suggest a possible involvement of DcR3 in the resistance to FasL-induced apoptosis displayed by the OCs from MM lytic bone disease.

0928

PATHOPHYSIOLOGICAL IMPLICATIONS OF POMALIDOMIDE IN TARGETING THE CLONOGENIC MULTIPLE MYELOMA SIDE POPULATION IN THE CONTEXT OF THE BONE MARROW MILIEU

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Background. Multiple myeloma (MM), a malignancy hallmarked by the accumulation of malignant plasma cells in the bone marrow, remains incurable despite the use of conventional and novel therapies. It has been proposed that currently established anti-MM regimens based on tumor regression may target and kill differentiated tumor cells, comprising the bulk of malignant plasma cells, while sparing the rare tumor-initiating and/or drug-resistant sub-populations, including presumptive MM stem cells. *Aims.* Therefore, the development of new strategies targeting these subpopulations of MM cells is important. *Methods.* and *Results.* In our study, we evaluated the existence of a MM population with "stem-like"features, known as side population (SP) cells, by flow cytometry-based Hoechst 33342 staining. SP cells exhibit substantial heterogeneity in MM cell lines and display higher mRNA expression and functional activity of ABCG2 transporter compared to non-SP cells. We observed evidence for high clonogenic potential of SP cells, as well as repopulation ability of SP cells. Moreover, SP cells had a high proliferation index compared to non-SP cells. Thalidomide and chemically-related IMiDs® drugs, such as lenalidomide, have in recent years become important agents for the treatment of MM. Another IMiD®, pomalidomide, is currently in clinical trials for the treatment of relapsed and refractory MM. In our study, pomalidomide significantly decreased the percentage and clonogenicity of SP cells at clinically relevant concentrations. Pomalidomide decreased ABCG2 transcript; induced activation of signaling pathways in SP cells (including phosphorylation changes in Akt, GSK-3 alfa/beta, MEK1, c-Jun, p53 and p70S6K). The bone marrow stromal cells (BMSCs) represent an important part of the bone marrow milieu, promote tumor cell growth and survival, and confer drug resistance against conventional agents. In our study, adherence to BMSCs increased the percentage, viability, and proliferation potential of SP cells. Interestingly, pomalidomide abrogated this stimulatory effect of stromal cells by significantly decreasing SP cell percentages. *Summary/Conclusions.* Our data show that pomalidomide targets and eliminates clonogenic SP cells, which raises the intriguing possibility that pomalidomide might represent a new class of cancer therapeutics targeting cancer stem cells. In addition, our data suggest that further studies of this tumor cell population in the context to bone marrow milieu are warranted towards the goal of developing new strategies and designing new cancer therapeutics to treat MM.

0929

BONE MARROW DERIVED MESENCHYMAL STEM CELLS ARE ATTRACTED BY A MULTIPLE MYELOMA CELL-PRODUCED CHEMOKINE (CCL25) AND STIMULATE MYELOMA CELL EXPANSION THROUGH AKT AND ERK PATHWAYS

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Background. Mesenchymal stem cells (MSCs) are multipotent stem cells that have immunomodulary properties and that produce a variety of hematopoiesis-stimulating cytokines. They can be expanded *in vitro* at clinical scale and are currently used in several pre-clinical and clinical studies for supporting hematopoiesis and/or controlling graft *vs.* host disease after hematopoietic stem cell transplantation, for tissue engineering or as gene therapy vehicles. MSCs have also been shown to have the capacity to home *in vivo* to tumor sites and to support tumor development by integrating into the tumor stroma. *Aims.* In this study we examined whether *in vitro* expanded bone-marrow-derived MSCs can be recruited by multiple myeloma (MM) cells and at which level MSCs directly affect growth and survival of MM cells. *Methods.* Human MSCs and murine MSCs were isolated from bone marrow aspirates of human voluntary donors and C57BL/KaLwRij mice, respectively. *in vitro* and *in vivo* migration assays were used to evaluate the migration ability of MSCs towards MM cells. Human stroma-dependent myeloma cells (MM5.1) , stroma-independent myeloma cells (RPMI8226) or murine stroma-dependent myeloma cells (5T33MMvv) and stroma-independent myeloma cells (5T33MMvt) were co-cultured with human or murine MSCs. The effect on proliferation/apoptosis was evaluated. Expression of proteins involved in cell cycle progression and apoptosis was tested by Western blot analysis. *in vivo* co-engraftment experiments (MSCs + MM cells) in the 5T33MM model were performed to confirm *in vitro* findings. *Results. in vitro* migration assays revealed that both human and murine MSCs can be attracted by MM cells. Accordingly we found by fluorescence microscopic analysis of paraffin sections from various tissues that DiI-labeled murine MSCs home in the 5T33MM model after intraveneous injection to MM-invaded organs (tibia and spleen) at a higher level as compared to the naive mice. We further identified CCL25 was the most important MM cell-produced chemokine that triggers MSC migration through the CCR9 receptor. By co-culturing MM cells and MSCs, we found that MSCs favor the proliferation of stroma-dependent MM cells by secreting soluble factors and by cell-cell contact, which was confirmed by co-engraftment experiments in the *in vivo* 5T33MM mouse model. We also demonstrated that MSCs protect *in vitro* MM cells against spontaneous and Bortezomib-induced apoptosis. Moreover we found that after co-culturing with mMSCs, murine MM cells show an up-regulation of phosphorylated AKT and ERK, accompanied with increased CyclinD2, CDK4 and Bcl-XL, and decreased cleaved caspase-3 and PARP. *Conclusions.* In conclusion our results provide evidence that reciprocal interaction between MSC and MM cells induce chemoattraction of MSCs by MM cells resulting in MSC-mediated stimulation of MM cell survival and proliferation. As precursors of the bone marrow stroma, MSCs seem to play an active role in the pathophysiology of this disease. Our data also suggest that therapeutical infusion of normal donor-derived MSCs should be considered with caution as these adult stem cells might enhance tumor progression *in vivo* resulting/contributing in disease relapse after MSC-based cytotherapy.

0930

MYELOID-DERIVED SUPPRESSOR CELLS IN PATIENTS WITH MULTIPLE MYELOMA AND MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE

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Background. Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of cells of myeloid origin, and they include immature macrophages, granulocytes, dendritic cells (DC) and other myeloid cells. In mice, they are phenotypically characterized as $CD11b^{+}Gr\text{-}1^{+}$. Humans MDSC have an immature phenotype, including lineage negative (Lin-), $CD14^{-}$, $HLA\text{-}DR^{-}$, $CD15^{+}$, $CD34^{+}$, $CD11b^{+}$, $CD33^{+}$, and $CD13^{+}$ cells. MDSC are considered an important tumor escape mechanism because they 1) impair T-cell functions through secretion immunosuppressive cytokines, perturbation of the arginine metabolism by inducible nitric oxide synthase (iNOS), up-regulation of reactive oxygen species (ROS), 2) promote tumor-dependent angiogenesis as well as tumor metastasis, and 3) provide tumor resistance to antiangiogenic drugs. Their accumulation has been described in the peripheral blood of patients affected by breast, lung, renal and neck carcinoma, melanoma, chronic infections, inflammatory diseases, and traumatic stress. We investigate MSDC in patients with multiple myeloma (MM) and monoclonal gammopathy undetermined significance (MGUS) by flow cytometry. *Methods.* We studied 36 patients with MM at diagnosis, 36 patients with MGUS, and 15 healthy controls (HC). *Results.* we observed that patients with MGUS showed approximately the same number of MDSC (CD11b+,CD13+,CD34+,CD14-,CD45+) in the peripheral blood compared to HC (2.26±1,66/mmc, *vs.*1,69± 0,87/mmc P=0,28). On the contrary, patients affected by MM showed a significant increase of MDSC *vs.* HC (4.09±3.07/mmc *vs.* 1,69± 0,87/mmc, P=0,008). *Conclusion.* Our results underlie the biologic difference between MM and MGUS and suggest that myeloid-derived suppressor cells could be involved in the progression of MGUS towards overt MM, through their immunosuppressive and proangiogenetic mechanisms.

0931

SERUM FREE LIGHT CHAINS ANALYSIS CAN EARLY DETECT RELAPSE/PROGRESSION IN INTACT IMMUNOGLOBULIN MULTIPLE MYELOMA

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Background. Serum free light chains (sFLC) analysis has proved its usefulness in disease diagnosis and early treatment response in multiple myeloma (MM). The sFLC ratio (sFLCR) has been included as one of the necessary criteria for achieving a "stringent complete response"in the proposed international response criteria. In contrast, performing this analysis during patient's follow up and after different treatments, in a daily practice setting, is still not very well defined yet. *Aim.* The main objective of this study was to assess the importance of sFLC analysis during MM patient's follow-up especially for relapse/progression detection comparing to concomitant traditional serum protein electrophoresis (sPE). *Methods.* We have analysed 174 MM patients diagnosed at the Edouard Herriot university hospital between years 2002 and 2008, for which a concomitant measurement of sFLC and sPE was done during follow-up. The sFLC analysis was performed using the Freelite® test from the Binding site on a BNII©, Dade Behring®, and for sPE, analysis was done using a Paragon CZE 2000® Beckman Coulter®. There were 92 (53%) males and 82 (47%) females, median age at diagnosis 57 years (34-72). One hundred and twenty patients (69%) had IgG (87K&33L), 52 (30%) IgA (41K&11L) and 2 (1%) IgD (1K&1L). Twenty six (15%) patients had renal insufficiency. We were interested to monitor the behaviour of sFLC and sPE in a way to early detect relapse or progression independently of treatment type. Slopes of the increase period corresponding to each measurement were compared using the student t-bilateral test with R statistical software (version 2.9.2). *Results.* After monitoring all patients, we observed 117 (67%) patients with relapse or disease progression and 57 (33%) patients were still in response to treatment. As our main objective was to detect relapse or progression, the 57 patients in response were excluded from the analysis. Among the 117 patients, in 77 (66%) cases, relapse or progression was detected by concomitant increase of both sFLC and the intact immunoglobulin level (iIg) with a significant earlier increase for sFLC (Figure1A). In 17 (15%) patients, the relapse or progression was characterised by the only increase of sFLC without any increase of the iIg (Figure1B). Contrarily, in 5 (4%) patients there was only an increase of the iIg without increasing the sFLC (Figure1D). Finally, in 18 (15%) patients, the relapse or progression was revealed by the increase of iIg faster than the concerned sFLC (Figure1C). When comparing slopes of increasing sFLC comparing to increasing iIg, we found a very high significant difference (P<0.001), thus showing that sFLC have a faster detection of relapse or progression. *Conclusion.* We have observed that in 81% (66%+15%) of patients, sFLC analysis enabled an earlier detection of relapse or progression compared to sPE, this could be very important for early treatment intervention especially for high risk patients. Since there are no uniform recommendations for the use of this analysis during follow-up, we recommend its concomitant use with sPE, waiting for guidelines.

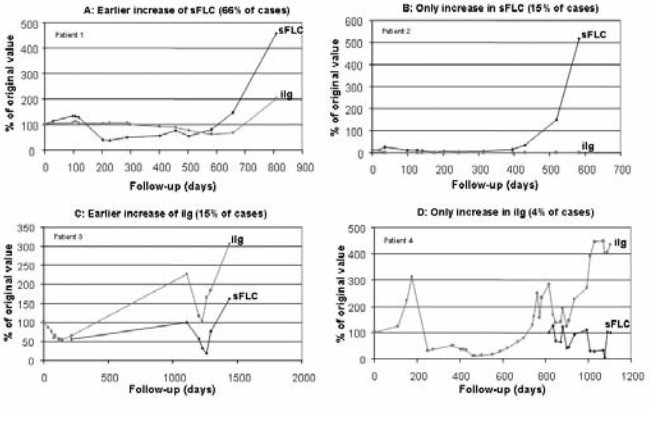

Figure 1. Different relapse/progression profiles.

0932

IMPACT OF THE MAGNITUDE OF FALL OF SERUM SOLUBLE SYNDECAN-1 (S-SYND-1) LEVELS AT PLATEAU PHASE, AFTER TREATMENT WITH BORTEZOMIB OR LENALIDOMIDE IN RELAPSED/REFRACTORY MULTIPLE MYELOMA (MM)

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Backgroud. Serum s-synd-1 levels at diagnosis constitute an independent prognostic factor in MM. In addition, the magnitude of its diminution from diagnosis to plateau phase after conventional induction treatment was shown to predict for patients' overall survival. Each time MM relapse after induction and is retreated, remission duration is shorter than the first time. However, new treatment modalities, bortezomib or lenalidomide - containing, have emerged for the management of relapsed/refractory MM patients, and they may improve patients' outcome after relapse, thus prolonging life expectancy. If s-synd-1 levels' decrease results in plateau phase or survival prolongation after treatment with new drugs (bortezomib, lenalidomide), is unknown. *Aims.* to evaluate the magnitude of serum s-synd-1 levels' reduction from relapse to plateau phase in a series of relapsed/refractory MM patients treated with bortezomib or lenalidomide. *Patients and methods.* Serum s-synd-1 was determined in 32 relapsed/refractory MM patients treated with Velcade and dexamethasone (VD) and 16 treated with Revlimid and dexamethasone (RD) before treatment induction and in plateau phase. In addition, s-synd-1 levels were also determined in 30's healthy individuals' sera (HI). s-synd-1 measurements were performed in duplicate by ELISA with a commercially available kit (Diaclone research, France). *Results.* MM type was IgG in 59% *vs.* 69% and IgA in 22% *vs.* 19% in the VD and RD group respectively. Light chain myeloma was present in 13% in both groups, 1 VD patient had IgD MM. More than 50% were in advanced ISS stage. The median time of relapses before treatment was 3 in both VD and RD groups. 90% of patients (including 44% nCR or better) responded in the VD group *vs.* 81% an the RD one (including 38% nCR or better). The median follow-up time from VD or RD treatment to relapse was 8.8 and 13.8 months respectively. At the time of relapse, before treatment, median s-synd levels in all VD and RD patients, were 90.5 ng/mL (10-1100) [median 105 for VD *vs.* 62ng/mL for RD] and became 22.5 ng/mL (7-650) [median 20 for VD *vs.* 26ng/mL for RD], while they were 27 ng/mL in HI (12-40). In the whole group, the achievement of >50% reduction of serum s-synd-1 from relapse to plateau was accompanied with increased plateau duration (median 4.9 months without *vs.* 16,6 months with s-synd-1 fall >50% at plateau, P=0.01). Increased plateau duration was also observed in all patients when a nCR or better was achieved (P=0.006). However, when the VD and RD group were analyzed separately, the quality of response obtained was more important for plateau duration in the VD group (P=0.001 *vs.* 0.65 in RD), while the decrease of s-synd-1 by >50% was more important in the RD group (P=0.01 *vs.* 0.12 for VD). With regard to overall survival after VD or RD treatment, only s-synd-1 decrease was important in the RD group (P=0.04, Figure 1). *Conclusions.* if confirmed in a larger series, our results suggest that the biologic modulation of lenalidomide implicates s-synd-1 and that there is a subgroup of patients that mostly benefit from this modality.

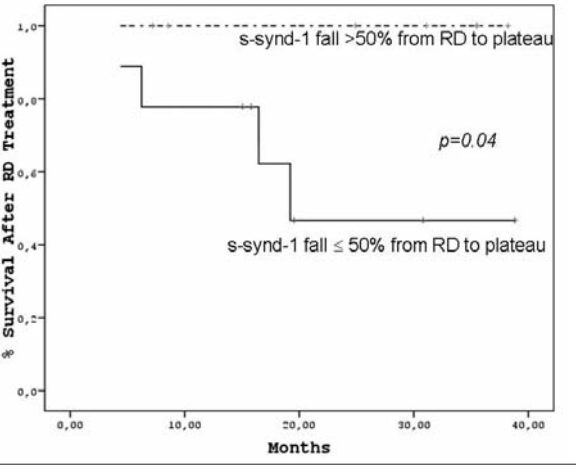

Figure 1.

0933

CLINICAL AND PROGNOSTIC SIGNIFICANCE OF ELEVATED SERUM BAFF LEVELS IN MULTIPLE MYELOMA

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Background. Multiple myeloma is characterized by accumulation of malignant plasma cells within the bone marrow in close contact with stromal cells, which secrete factors/cytokines promoting tumour cell growth and survival. Among these factors B-cell-activating factor (BAFF) is a member of the tumour necrosis factor superfamily and is expressed by a number of cell types. BAFF is a critical survival/maturation factor for peripheral B cells and this activity is mediated through a BAFF-specific receptor, BAFF-R. Over expression of BAFF has been linked to autoimmune disease and B cell neoplasia. *Aims.* The purpose of this study was to measure the serum levels of BAFF in multiple myeloma and to assess whether it could be a strong indicator of the magnitude of angiogenesis, disease severity and the patients' survival. *Methods.* 48 patients with multiple myeloma and 12 healthy controls were studied. Patients were staged with Durie-Salmon system. Serum samples were collected from all patients at the diagnosis and at the plateau phase after effective treatment (n=18). We have also performed bone marrow biopsies prior to treatment. Microvascular density was measured by staining bone marrow vessels with anti-CD31. At the diagnosis serum levels of BAFF, PCNA and TNFα were determined by ELISA, as well as LDH levels. BAFF was also measured at plateau phase. *Results.* BAFF's serum levels were significantly higher in myeloma patients compared to control (P<0.001) (988.6±720.3pg/mL *vs.* 279.8±164.3pg/mL). Significantly serum BAFF levels were proportionally increased in accordance with the disease progression (P<0.001). (Stage I:481.1±188.2pg/mL, stage II:754.9± 245.9pg/mL and stage III:1394.1±772.8pg/mL). BAFF's serum levels showed significant correlation with LDH concentration (r= 0.273, P<0.06), the PCNA levels (r=0.578, P<0.001), TNFα (r=0.495, P<0.001) and bone marrow microvascular density (r=0.303, P<0.04). BAFF levels decreased significantly after effective chemotherapy reaching values comparable to healthy controls (values pre and post treatment were 849.3±786.0 pg/mL *vs.* 281.8±174.1pg/mL, respectively) (P<0.001). Analysis of the patients' survival (Kaplan Mayer method) showed that levels >948.92 pg/mL had lower survival rates (P<0.001). *Conclusions.* BAFF serum levels were significantly elevated in patients with multiple myeloma suggesting that BAFF may be involved in the pathogenesis of the disease. Increased levels are correlated with disease activity increased angiogenesis and poor prognosis in MM.

0934

OLIGOCLONAL BANDS IN PATIENTS WITH MULTIPLE MYELOMA IN COMPLETE RESPONSE AFTER INDUCTION CHEMOTHERAPY: ASSOCIATION WITH THE USE OF NOVEL AGENTS

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Background. The emergence of oligoclonal bands is a well recognized event after autologous stem cell transplantation in multiple myeloma (MM). These atypical serum immunofixation (IFE) patterns are associated with good prognosis, likely due to a durable immune reconstitution. They can appear with novel immunomodulatory drugs such as lenalidomide, but its prevalence with other induction treatments has not been reported. *Aims.* To determine the prevalence of serum and/or urine oligoclonal bands in patients with MM in CR after primary therapy with cytotoxic agents or new induction chemotherapy regimens incorporating novel drugs up-front. *Methods.* Thirty-three patients (15M/18F; median age at diagnosis 59 years, range 25 to 89) with MM in CR after different induction regimens were studied. Initial baseline demographics, clinical and laboratory data, treatment and follow-up were collected. An oligoclonal band was defined by the presence of a serum and/or urine IFE monoclonal spike different either in heavy and/or light chain component from the original myeloma protein. *Results.* The initial clinical and laboratory findings as well as the induction treatments are shown in the table. Eighteen patients (54.5%) received induction with conventional chemotherapy and 15 with novel agents (45.5%). In the latter group, the induction regimen was based on combinations of glucocorticoids with lenalidomide (26.7%), thalidomide (26.7%), bortezomib (33.3%) or bortezomib plus lenalidomide (13.3%). In the overall series, 11 out of the 33 patients (33.3%) developed an oligoclonal band. In the group treated with cytotoxic agents, the prevalence of oligoclonal bands was observed in 2 patients (11.1%), both of them had been treated with cyclophosphamide and dexamethasone. In contrast, induction with novel drugs resulted in 9 out of 15 patients (60%) developing oligoclonal bands in serum and/or urine (chi-square test 2-sided, P=0.003). *Conclusions.* This is the first report showing a significant different frequency of oligoclonal bands in patients with MM in CR after conventional cytotoxic therapy *vs.* induction incorporating novel agents. This difference is likely due to a strongest immune reconstitution resulting from the effect of novel drugs. However, the prognostic impact of the presence of oligoclonal bands emerging after induction therapy is still unknown.

Table.

Variable	Total group (n=33)	Cytotoxic drugs group (n=18)	Novel drugs group (n=15)
Median age, y (range)	59 (25-89)	57.9 (25-89)	61.4 (47-79)
Male/female	15/18	8/10	7/8
Heavy-chain component (%)			
IgG	27.3	16.7	40
IgA	27.3	27.8	26.7
Only light chains	30.3	38.9	20
IgD	12.1	16.7	6.7
IgM	3	0	6.7
Light-chain component (%)			
κ	48.5	50	66.7
λ	51.5	50	33.3

0935

MULTIDRUG RESISTANCE GENE EXPRESSION IN PLASMA CELL MYELOMA

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Background. Multi-drug resistance (MDR) is a phenomenon that can lead to impaired treatment efficacy in all forms of malignancy. The most prominent forms of MDR are mediated by the drug effluxing actions of certain adenosine tri-phosphate binding cassette (ABC) transport proteins. Recent debates in plasma cell myeloma (PCM) have questioned which of the many ABC transporters are to be implicated in the course of this disease and also in the treatment of PCM post relapse. rs1045642 is a SNP within *ABCB1* which is thought to affect the activity of the protein product (P-glycoprotein) and has demonstrated significant associations with overall survival in PCM1. *Aims.* This study was designed to investigate the expression of the MDR associated genes; *ABCB1*, *ABCB4*, *LRP*, *ABCC1* and *ABCG2* in PCM. We also wished to determine the impact of rs1045642 on *ABCB1* mRNA levels in PCM. *Methods.* 89 patients with a confirmed diagnosis of PCM were studied with ethical consent approved via ORECNI. Of these patients, 81 were biopsied at diagnosis of either MGUS or MM and 8 at relapse. Tumour cells were separated from bone marrow biopsies using $CD138^+$ magnetic microbeads and RNA was extracted using Trizol. mRNA expression was determined for each gene using relative quantification and taqman validated assays. Results are expressed as a fold change from expression in normal plasma cells. *Results.* Of the 5 genes investigated, *ABCB4* (MDR-3) has demonstrated the greatest increase in PCM patients at diagnosis with a mean fold change of 26. In relapsed samples, all genes apart from *ABCB1* have demonstrated a substantial decrease in mean fold change with *ABCB4* mean fold change reduced to 9. *ABCB1* mean fold change was increased from 0.6 at diagnosis to 2.8 in relapsed samples. MDR gene expression was not significantly associated with currently available prognostic factors in PCM (B2M, Albumin, Ig class and light chain restriction). rs1045642 does not affect *ABCB1* mRNA levels in PCM patient samples. *Conclusion.* This study suggests that *ABCB4* may be the most prominent contributor to MDR and initial treatment failure in newly diagnosed PCM. *ABCB1*, however, appears to have

increased mRNA levels in relapsed samples suggesting that P-gp may be a more prominent contributor to treatment strategies, post relapse, in PCM. MDR gene expression may be an independent factor affecting treatment within this patient cohort. rs1045642 does not alter *ABCB1* mRNA levels in PCM suggesting that effects of this SNP manifest at the protein level. Further work will investigate the expression and activity of P-glycoprotein in PCM and will determine the clinical effect of rs1045642 on P-gp.

References

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0936

FLOW CYTOMETRIC IMMUNOPHENOTYPIC CHARACTERISTIC OF 36 CASES OF PLASMA CELL LEUKEMIA

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Aim of the study was to determine expression of adhesion molecules CD11a/CD18 (LFA1), CD11b, CD29/CD49d (VLA-4), CD44 (H-CAM), CD54 (ICAM-1), CD56(N-CAM) and CD71(Tr1R), CD117 (c-kit), CD126 (IL-6R), on peripheral blood (PB) and bone marrow (BM) plasma cells in 36 plasma cell leukemia (PCL) patients (23 primary, 13 secondary) at diagnosis and in 47 multiple myeloma (MM) patients. Immunophenotyping was performed simultaneously on freshly collected PB and BM samples using flow cytometry. Plasma cells were identified as cells showing high-density expression of CD38, expression of CD138 (syndecan-1) and CD45 low /-. Results of analysis were presented as relative values of numbers of cells with antigen expression and as relative fluorescence indices (RFIs) of studied antigens. BM plasma cells showed expression of particular antigens in a following proportion of cases: CD49d 100%, CD29 94%, CD54 93%, CD44 83%, CD56 60%, CD18 26%, CD11b 29%, CD11a 19%, CD117 27%, CD71 30%, CD126 100% while expression of those antigens on PB plasma cells was present in following percentage of patients: CD49d 100%, CD29 96%, CD54 93%, CD44 95%, CD56 56%, CD18 50%, CD11b 53%, CD11a 29%, CD117 26%, CD71 28%, CD126 100%. Expression of CD54 (BM 71±31%, RFI 16±4; PB 72±3%, RFI 16± 3) was significantly higher than that of adhesion molecules belonging to integrin β2 family: CD11a, CD18 and CD11b, on both BM and PB cells (P<0.01). Expression of CD18, CD11a and CD11b was differential between two cell compartments: lower on BM and higher on PB cells. CD11a - BM 13±17%, RFI 14±3; PB 26±25%, RFI 18.7±3.7, CD18- BM 16±16%, RFI 12.8±3.4; PB 36±20%, RFI 16.2±1.5, CD11b - BM 16±24%, RFI 17±4; PB 33±26%, RFI 20±3. Plasma cells in BM of PCL patients showed significantly greater granularity (786±253) and size (1439±266) than those in PB (554±191) and (1308±186) (P=0.0001 and P=0.04, respectively). However, no differences in cell size and granularity were revealed between BM plasma cells from PCL patients and MM patients (granularity 833±260; size 1414±240) (P=0.249 and P=0.659, respectively). The difference in the rate of BM CD44+ cells between PCL patients (79±33%) and MM patients (46±36%) was significant (P=0.0062). In 55% of primary PCL cases malignant plasma cells did not express CD56 while incidence of CD56 positivity in secondary PCL did not differ from that observed in MM cases (67±37%). *Conclusions.* Immunophenotype of leukemic plasma cells characterizes mainly increased expression of CD38, CD54 and CD138, expression of CD29, CD49d, CD44, CD126 and disturbed expression of CD18, CD11a and CD11b. In one third of cases, tumor cells show expression of CD56, CD71 and CD117. Impaired expression of adhesion molecules such as CD11a/CD18 or CD56 may explain hematogenic dissemination characterizing PCL. Adhesion molecule expression according to proportion of plasma cells expressing a given antigen in PB and BM presents a following pattern (in diminishing order): CD49d>CD44>CD54>CD29>CD56>CD18 >CD11b>CD11a. Immunophenotyping of plasma cells in PCL, alike in MM, might be useful for detecting minimal residual disease in cases with aberrant antigen expression and for selection of therapeutic agents towards specific membrane targets.

0937

CHANGES IN THE EXPRESSION OF TELOMERE-ASSOCIATED GENES IN MULTIPLE MYELOMA AND MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE. CORRELATION WITH CLINICO-PATHOLOGICAL PARAMETERS

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Background. Telomere-binding proteins protect chromosome ends through telomere end-capping and length control. Several studies have investigated the expression of telomerase and telomeric proteins in different lymphoid neoplasms, but little is known about its expression profile in plasma cell disorders. *Aims.* In this study, we have evaluated the mRNA levels of TRF1, TRF2, TANK1 and hTERT genes and telomere length (TL) in patients with multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS). Results were correlated with clinico-pathological characteristics. *Methods.* mRNA samples from 132 patients: 68 with MM (33 males; mean age: 68.5 years; range: 30-87 years; 35% ISS stage III) and 64 with MGUS (28 males; mean age: 69.3 years; range: 39-88 years) and 15 normal controls (NC) were studied. All patients gave informed consent and the study was approved by the Ethics Committee of our Institution. Gene expression was quantified on mRNA samples from patients and controls by real time PCR, using TaqMan assay. GAPDH was used as housekeeping gene to normalize the expression of each target gene. TL measurements were performed by Terminal Restriction Fragments. The proliferative index was evaluated by immunohistochemistry with Ki67 antibody. For statistical analysis, Mann-Whitney test, Kendall´s coefficient and receiver operating characteristic (ROC) curves were used. *Results.* Differences in the expression of telomeric genes was observed in patients compared to NC (Figure 1). Significant differences in mRNA levels of TRF1, TRF2 and TANK1 between MGUS and MM were found. TRF1 showed lower levels (P<0.006) meanwhile higher levels for TRF2 and TANK1 (P<0.005) in MM respect to MGUS were detected. In both entities, an up-regulation of hTERT and a positive association between TRF2-TANK1, TRF2-hTERT and TANK1-hTERT (P<0.01) was observed. Patients showed a wide spreading of hTERT values. For a better analysis, hTERT levels were divided into three groups taking into account the cut-off points generated by ROC analysis: low (GI:≤1.08), intermediate (GII: <1.08 y ≥5.0) and high (GIII: >5.0). In both pathologies, a similar distribution of patients per group was observed, with most of cases in GI (70%) and the less proportion in GIII (9%). In MM, higher expression of TRF2 (0.22±0.03) and TANK1 (0.77±0.16) in GIII respect to GI (TRF2: 0.06±0.01; TANK1: 0.28±0.07) and GII (TRF2: 0.07±0.01; TANK1: 0.37±0.05) (P<0.01) was observed, while increased TRF2 levels in GIII (0.10±0.02) compared to GI (0.04±0.004) in MGUS (P<0.01) was found. TL had a significant reduction in patients respect to NC (P≤0.04). In both pathologies, GIII showed the shortest TL (MM: 5.28±2.56kb; MGUS: 4.90±1.75kb). In MM, the percentage of bone marrow infiltration was positively associated with TRF2, TANK1 and hTERT expression (P≤0.005) and negatively with TL (P=0.02), whereas LDH was significantly correlated with TRF2 mRNA (P=0.008). Ki-67 index was positively associated with TRF2, TANK1 and hTERT expression (P≤0.03) and negatively with TL (P=0.002). *Conclusions.* The differential expression pattern observed suggest that changes in telomeric proteins composition may be associated to the establishment and maintenance of short telomeres in MGUS and MM patients, contributing to tumour progression.

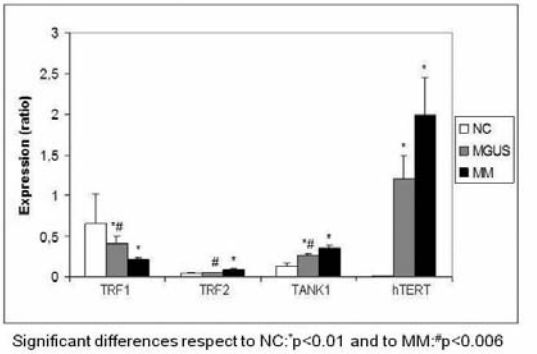

Figure 1. Expression of telomeric genes.

0938

FLOW CYTOMETRY EVALUATION OF BONE MARROW PLASMACELLS IN MONOCLONAL GAMMOPATHY OF UNCERTAIN SIGNIFICANCE (MGUS): CORRELATION WITH MONOCLONAL COMPONENT, FREE LIGHT CHAIN AND MAGNETIC RESONANCE IMAGING

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Background. While neoplastic plasmacells (PCs) retain some of the phenotypic characteristics of normal PCs, including strong CD38 expression, immunophenotyping studies demonstrated that abnormal PCs lack CD19 and express CD56 antigens. Assessment of serum free light chain (sFLC) concentrations is a powerful tool to qualify response stringency and duration in multiple myeloma. The presence of an abnormal sFLC ratio also represents a risk factor for progression in different types of plasmacell dyscrasias. *Aim.* The rationale of this study was to detect the incidence of the abnormal PC phenotype in MGUS compared to polyclonal plasmacytosis as a control cohort. We also addressed whether a BMPC phenotype could differentiate subsets of MGUS cases with different amounts of sMC or abnormal FLC k/L ratio, or cases with pathological MR imaging of the axial skeleton. *Methods.* One hundred MGUS and 68 plasmacytosis were analyzed. BMPCs were identified by their characteristic light scatter distribution and a sequential gating strategy, CD19, CD45, CD38 and CD56 reactivity were assessed using an EPICS Profile II flow cytometer (Coulter Electronics, Inc., Hiealeah, FL). sFLCs were determined using a particle enhanced, high-specificity, homogeneous FLC immunoassay [Freelite; The Binding Site, Birmingham, UK; performed on a Delta Nephelometer (Radim, Milano, Italy)]. *Results.* Considering the lowest CD19 (median 63.4%; range 22.9-93.5) and the highest CD56 (median 63.4%; range 22.9-93.5) values expressed in the 68 benign plasmacytosis, we established 20% as the best cut-off value for CD19 and CD56 to differentiate normal from abnormal BMPC. Thus, MGUS cases were divided into 3 groups: normal BMPC (CD19high/CD56low, 26 cases), abnormal BMPC ($CD19^{low}/CD56^{high}$, 24 cases) and BMPC with an intermediate phenotype (34 cases; CD19low/CD56low, 24 cases; $CD19^{high}/CD56^{high}$, 10 cases). The mean sMC level was 1.01+0.06 gr/dL. A significant direct correlation was demonstrated between BMPC detected by flow (CD45neg/CD38pos) (Rho=0.3, P=0.003) and sMC. Notably, the expression of BMPC CD19 (RHO=-0.44, P<0.0001), but not CD56, inversely correlated with sMC. The mean value of sMC was lower (P=0.002) for cases expressing CD19>20% on BMPC (0.9+0.1) *vs.* those cases with an expression of CD19<20% (1.2+0.1). Moreover, a trend towards a higher amount of sMC was detected in the abnormal phenotype group. sFLC k/L ratio was investigated in 39 MGUS cases (21 abnormal). An abnormal sFLC ratio was associated with significantly lower percentage (P=0.019) of CD19+ BMPCs [16.7(range 2.7-76) *vs.* 6.9 (range 0.1-76)], while no significant association was documented with CD56 expression. Of the 21 cases with abnormal sFLC ratio, only 2 (9.5%) had a $CD19^{high}/CD56^{low}$, 10 (47.6%) a $CD19^{low}/CD56^{high}$, and 9 an intermediate BMPC phenotype. MR imaging of the axial skeleton was carried out in 55 cases. All 4 patients showing a pathologic MR pattern had an abnormal ($CD19^{low}/CD56^{high}$) BMPC pattern. However, the remaining normal MR cases were equally distributed within the different phenotypes. *Conclusion.* Our results indicate that Flow-Cytometry analysis may detect MGUS cases with different BMPC phenotypes associated with amounts of sMC and sFLC k/L ratio. Whether the presence of such a phenotype in MGUS cases may have an impact in predicting a different clinical outcome should be evaluated.

0939

DETECTING MYELOMA - WAYS TO SHORTENING AN OFTEN PAINFUL AND TEDIOUS PATIENT ODYSSEY: RESULTS FROM AN INTERNATIONAL SURVEY CONDUCTED BY MYELOMA EURONET, THE EUROPEAN NETWORK OF MYELOMA PATIENT GROUPS

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Background. It is well understood that a prolonged delay in myeloma diagnosis has a significant impact on disease-free survival. Myeloma can have various non-specific symptoms such as back pain, bone pain, bone fractures, recurrent infections, tiredness/weakness, and kidney problems. Patients therefore present to a range of medical professionals before their myeloma is detected, also including general practitioners/family doctors (GP/FD) and orthopaedic surgeons/traumatologists (OS/T). *Aims.* The aims of the study were to obtain information about the path patients take to myeloma diagnosis, what non-haematological/non-oncological medical professionals do to detect myeloma, the time necessary to detect myeloma, and steps required to avoid delays in myeloma diagnosis. *Methods.* A survey of 303 physicians from 56 countries (91.4% European), including 90 GP/FD (29.7%) and 206 OS/T (68.0%), was conducted via self-administered questionnaire including ten multiple-choice questions. In addition, 349 myeloma patients (MP) and myeloma patient relatives (MPR) from 37 countries of treatment (90.3% European), including 239 MP (68.5%) and 110 MPR (31.5%), completed a corresponding questionnaire with nine multiple-choice questions. *Results.* MP/MPR (n=236/109) stated the most frequent symptoms experienced before initial doctor consultation were back pain (45.8%/59.6%), tiredness/weakness (35.2%/37.6%), bone pain (26.7%/36.7%), recurrent infections (16.5%/17.4%), shortness of breath (14.4%/13.8%) and bone fractures (11.4%/11.9%). As a result, the most frequently consulted doctors according to MP/MPR (n=236/108) were GP/FD (64.4%/60.2%), haematologists (8.1%/5.6%) and OS (6.4%/5.6%). From there, MP/MPR (n=238/106) stated, the most frequent referrals were to haematologists (41.6%/24.5%), oncologists (10.5%/11.3%), OS (9.7%/11.3%), GP/FD (6.7%/7.5%) and rheumatologists (5.5%/6.6%). GP/FD (n=90) usually treat back pain (88.9%), tiredness/weakness, bone pain, shortness of breath (60.0% each), and recurrent infections (58.9%); OS/T (n=206) usually treat bone fractures (82.4%), bone pain (68.9%) and back pain (67.5%). Confronted with these symptoms, routine tests by GP/FD (n=89) include blood test (93.3%), x-ray of bones (75.3%) or urine test (68.5%); OS/T (n=204) would do x-ray of bones (90.7%), blood test (73.0%), MRI (51.0%) or CT scan (45.6%). However, 65.9% of GP/FD (n=88) and 47.5% of OS/T (n=202) were not very familiar/not familiar at all with myeloma, 80.7% of GP/FD (n=88) and 63.3% of OS/T (n=199) rarely/never detected myeloma, and 60.7% of GP/FD (n=89) and 46.8% of OS/T (n=201) rarely/never referred patients to myeloma specialists. MP/MPR (n=230/108) stated that myeloma is mainly detected by haematologists (47.0%/47.2%), GP/FD (13.5%/10.2%), oncologists (8.3%/11.1%) and OS (6.1%/3.7%). According to MP/MPR (n=200/104), 44.5%/63.5% of patients received treatment for one/more symptoms before myeloma detection, including (n=89/66) pain treatment (33.7%/42.4%), physiotherapy/chiropractor/osteopath treatment (14.6%/21.2%) and orthopaedic interventions (11.2%/13.6%). According to MP/MPR (n=229/109), 76.0%/63.3% of patients saw 1-3 doctors before myeloma detection, and 23.1%/34.9% of MP/MPR stated it took 4 doctors or more to detect myeloma. According to MP/MPR (n=231/108), the average time for detecting myeloma is 186.8 days. According to GP/FD (n=89), OS/T (n=196) and MP/MPR (n=217/109), the most important steps to avoid delays in myeloma diagnosis are better information for (75.3%/71.9%/57.1%/67.9%), and better education of, medical professionals (53.9%/45.9%/40.1%/49.5%). *Summary/Conclusions.* Little awareness of myeloma among GP/FD and OS/T most likely contributes to delays in myeloma diagnosis. Related information and education should concentrate on GP/FD.

0939a

LYMPHOCYTE ABNORMALITIES WITHIN GAUCHER DISEASE MAY PREDISPOSE TO AN ELEVATED RISK OF HAEMATOLOGICAL MALIGNANCY

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Background. Gaucher disease (GD), the most common lysosomal storage disorder, secondary to an inherited deficiency of glucocerebrosidase. GD is associated with an increased incidence of polyclonal gammopathy, monoclonal gammopathy of undetermined significance and multiple myeloma. Monocyte-derived lipid laden macrophages or Gaucher cells are the cellular hallmark of Gaucher disease but relatively little is known about lymphocyte abnormalities in this condition. *Aims.* To demonstrate numerical and functional abnormalities of lymphocytes from GD patients. *Methods.* Patients with GD were recruited from the Royal Free Hospital Lysosomal Storage Disorders Unit together with age-matched healthy controls. Lysosomal glucocerebrosidase activity of peripheral blood mononuclear cells was determined by flow cytometry, utilizing the substrate fluorescein di-β-glucopyranoside (FDGlu). Membrane dysfunction was assessed by determining co-localization of lysotracker-red and BODIPY – lactosylceramide by fluorescent microscopy. Lymphocyte subsets were identified by immunostaining of CD19, CD4, CD8, CD56, 6B11 and CD3. Killing assays were performed using various effector:target ratios of IL-2 stimulated peripheral blood mononuclear cells (effector cells) and PKH26 labeled-K562 cells (target cells). The nuclear stain TO-PRO-3 iodide was used as a marker of cell death. *Results.* There was no difference in the proportion of lymphocytes in controls, treated GD patients or untreated GD patients. Membrane dysfunction was demonstrated in GD lymphocytes by accumulation of BODIPY within lysosomes. Glucocerebrosidase activity was significantly less in GD lymphocytes ($P<0.01$), CD4 T-cells ($P<0.01$), CD8 T-cells ($P<0.01$), NK-T cells ($P<0.05$), NK-T like cells ($P<0.05$) and B-cells ($P<0.01$) compared to controls. Immunophenotyping revealed a reduced percentage of CD4 lymphocytes ($P<0.05$), CD3-veCD56+ve NK-T cells ($P<0.05$) and CD3+ve6B11+ve invariant NK-T cells ($P<0.01$) compared to controls. There was an elevated number of CD3+veCD56+ve NK-T like cells ($P<0.01$) and a significantly lowered CD4:CD8 ratio ($P<0.0001$) in GD patients. Further functional anomalies demonstrated enhanced killing of the K562 myeloid cell line by control peripheral blood mononuclear cells compared to GD patients in killing assays at effector:target ratios of 40:1 ($P<0.01$), 20:1 ($P<0.05$) and 10:1 ($P<0.05$). *Conclusion.* GD lymphocyte subsets have statistically less glucocerebrosidase activity than controls and have demonstrable membrane dysfunction. Differences in the lymphocyte profile including a low CD4:CD8 ratio plus reduced NK-T and invariant NK-T cell numbers may contribute to an elevated risk of malignancy. Killing assays additionally support the impaired functionality of GD lymphocytes and raise the question of decreased tumor surveillance.

Myeloma and other monoclonal gammopathies - Clinical 3

0940

LENALIDOMIDE IN COMBINATION WITH MELPHALAN AND DEXAMETHASONE IN PATIENTS WITH NEWLY-DIAGNOSED LIGHT-CHAIN (AL)-AMYLOIDOSIS: A MULTICENTER PHASE I/II DOSE ESCALATION STUDY

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Introduction. The combination of melphalan and dexamethasone (M-dex) is widely used in patients with newly diagnosed AL-amyloidosis, with hematologic and organ response rates of 50 and 40%, respectively (Jaccard, New Engl J Med 2007). Lenalidomide has also been evaluated, mainly in the relapse setting, and the initial dose of 25 mg/day was poorly tolerated. However, a 15 mg/day dose regimen was well tolerated and effective, with both hematologic and organ responses (Dispenzieri, Blood 2007 & Sanchorawala, Blood 2007). Combining M-dex with lenalidomide could increase the response rate but the toxicity of this regimen is still unknown. Thus we have initiated a multicenter single-arm open-label phase I/II dose escalation study of lenalidomide administered in combination with M-dex. *Patients and methods.* The primary endpoint was the incidence of dose limiting toxicities (DLT) during the first cycle of lenalidomide at a given dose level in order to determine the maximum tolerated dose (MTD). In addition to melphalan 0.18 mg/kg/day from day 1-4 of each 28 day cycle and dexamethasone 40 mg/day from day 1- 4 of each 28 day cycle, patients were successively exposed to escalating doses of lenalidomide (5, 10, 15 and 20 mg once daily on days 1-21 of a 28 day cycle). Nine cycles were planned at each dose level, according to safety and efficacy. DLT was defined using National Cancer Institute common toxicity criteria during the first 4 weeks of treatment (one cycle) as the following: at least grade 2 cardiac arrhythmia, at least grade 3 non hematologic toxicity, grade 4 neutropenia lasting >7 days or any other Grade 4 hematologic toxicity, or treatment delay due to toxicity that occurred during the first cycle. Organ involvement and the response to therapy were evaluated according to the international consensus guidelines (Gertz Am J Hem 2005). *Results.* From 03/2008 to 01/2009, 27 patients were enrolled. 1 patient withdrew consent after the first month of therapy and was thereafter lost to follow-up. No DLT was observed among the patients treated at 5 (3 patients), 10 (4 patients) and 15 mg lenalidomide/day (12 patients). 4 / 7 patients treated in cohort 4 at the dose of 20 mg lenalidomide/day experienced DLT, grade 4 hematologic toxicity and treatment delay due to toxicity; thus 15 mg lenalidomide/day, in combination with M-dex, was considered as the MTD. With a median follow-up of 16 months, 21/26 patients (80.8%) are alive. Five deaths were observed, due to progressive disease in 4 cases (median time from diagnosis 1 month, 1 to 3), or cholangiocarcinoma in 1 case (7 months after the diagnosis of AL-amyloidosis). Hematologic and organ responses were observed in 58 and 50% of the cases, respectively. At the reference date of Feb1st, 2010 none of the responding patients experienced progression, and all were able to receive 9 cycles according to the protocol. *Conclusion.* The recommended dose of lenalidomide in combination with M-dex in subjects with AL-amyloidosis previously untreated is 15 mg/day (days 1-21 of a 28 day cycle). Response and survival rates compare favourably with those achieved with M-dex alone. A prospective phase III trial comparing M-dex with M-dex lenalidomide should be valuable.

0941

PHASE III STUDY OF ENOXAPARIN VERSUS ASPIRIN VERSUS LOW-DOSE WARFARIN AS THROMBOPROPHYLAXIS FOR PATIENTS WITH NEWLY DIAGNOSED MULTIPLE MYELOMA TREATED UPFRONT WITH THALIDOMIDE-CONTAINING REGIMENS

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Thalidomide as part of upfront therapy for multiple myeloma (MM) is associated with an increased risk of venous thromboembolism (VTE) which necessitates routine prophylaxis. Controversies exist concerning the best thromboprophylactic regimen to be used in these patients. To address this issue, we designed a phase III study aimed at prospectively comparing low molecular weight heparin (LMWH) (Enoxaparin 40 mg/d) with low-dose aspirin (ASA) (100 mg/d) with fixed low-dose warfarin (WAR) (1.25 mg/d) in MM patients treated with regimens incorporating thalidomide. Nine hundred and ninety one patients with newly diagnosed MM were primarily randomized to receive the following induction treatments: 1) Velcade-Thalidomide-Dexamethasone (VTD) (Velcade, 1.3 mg/m^2 twice weekly for 2 weeks; Thalidomide, 200 mg/d; Dexamethasone, 320 mg per cycle); 2) Thalidomide-Dexamethasone (TD) (Thalidomide 200 mg/d; Dexamethasone 320 mg per cycle); 3) Velcade-Melphalan-Prednisone (VMP) (Velcade, 1.3 mg/m^2 twice weekly for 2 weeks; Melphalan, 9 mg/m^2 d 1-4; Prednisone, 60 mg/m^2 d 1-4); 4) Velcade-Melphalan-Prednisone-Thalidomide (VMPT) (Velcade, Melphalan and Prednisone as in VMP, with added Talidomide, 50 mg/d). Patients randomly assigned to VTD or TD or VMPT were subsequently randomized to receive anticoagulation prophylaxis with either LMWH or ASA or WAR. By the opposite, patients randomized to Velcade-Melphalan-Prednisone (VMP) did not receive any prophylaxis and were used as controls. Study end points included incidence of VTE, acute cardiovascular events, sudden death, bleeding and any other serious adverse events. The frequency of major risk factors for VTE was similar in the three groups of patients receiving different antithrombotic prophylaxis. The incidence of VTE was 5% in the LMWH group, 6% in the ASA group and 8% in the WAR group (p not significant). The corresponding value in the VMP group was 2%. Median times to onset of VTE for patients who received LMWH or ASA or WAR were 4.7, 2.4 and 2.4 months, respectively. Patients treated with VTD and VMPT had a lower, albeit not statistically significant (P=0.08), incidence of VTE (5%) in comparison with those on TD (8%). Cardiovascular events with LMWH, ASA and WAR were 2%, 1% and 0.5%, respectively. The risk of bleeding events was higher with ASA (3%) in comparison with WAR (1%) (p not significant). The incidence of combined thrombosis, bleeding and cardiovascular events was 9% in the LMWH group, 10% in the ASA group and 9.5% in the WAR group (p not significant). In conclusion, the overall incidence of VTE in the three thromboprophylactic groups was less than 10%, a value not superior to that expected during the natural course of MM. LMWH, ASA and WAR are likely to be effective thromboprophylactic treatments in standard-risk MM patients receiving thalidomide-based induction regimens.

0942

IMPROVED PROGRESSION-FREE SURVIVAL AND OVERALL SURVIVAL WITH THALIDOMIDE MAINTENANCE THERAPY IN MULTIPLE MYELOMA: A META-ANALYIS OF RANDOMIZED TRIALS IN 2274 PATIENTS

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Background. A meta-analysis (Hicks *et al*. Cancer Treat Rev 2008) from four randomized trials testing maintenance thalidomide in patients with multiple myeloma (MM) showed no significant improvement in overall survival (OS). After publication of the article, one of the included trials was retracted and new studies or updates of previous data were published. *Aims.* A new meta-analysis was performed to re-evaluate the influence of thalidomide maintenance on OS in patients with MM. In addition, we also analysed progression-free survival (PFS) and toxicity. *Methods.* PubMed, the Cochrane Library and conference proceedings from ASH, ASCO, IMW, and EHA were searched using the headings "myeloma"and "thalidomide", lastly in January 2010. Studies were included if they were randomized controlled trials (RCTs) of patients with MM receiving thalidomide maintenance treatment as monotherapy or combination therapy. Data were pooled using the random effects model. Measures of treatment effect were hazard ratios (HR) for survival data and relative risk (RR) for toxicity. When not available from the articles, HRs were estimated using the methods of Parmar *et al.*. (Statist Med 1998). *Results.* Nine RCTs of thalidomide maintenance therapy (5 published as full papers, 4 in abstract form) were identified. Three trials were excluded because no survival data were reported. The six remaining trials included 2274 patients and compared thalidomide as monotherapy (one trial) or combination therapy (corticosteroids two trials, corticosteroids + interferon one trial, interferon one trial and pamidronate one trial) with interferon (2 trials), interferon + dexamethasone (2 trials), prednisolone (one trial) or no maintenance (one trial). Five trials included patients with newly diagnosed MM, one trial included newly diagnosed and relapsed/refractory patients. Prior therapy was autologous stem cell transplantation (four trials) or conventional chemotherapy (two trials). In three trials thalidomide was also part of the induction regimen. PFS was significantly improved with maintenance thalidomide (HR 0.64, 95%CI 0.56-0.73, P<0.001). The effect was similar in trials with or without prior thalidomide induction treatment. Interestingly, OS was also improved with maintenance thalidomide (HR 0.70, 95%CI 0.55-0.89, P=0.004). In the subgroup of trials with prior thalidomide induction, the effect did not reach significance (HR 0.78, 95%CI 0.60-1.01, P=0.06). No significant heterogeneity among all RCTs existed between PFS or OS HRs (I^2=23%, P=0.26 and I^2=48%, P=0.09 respectively). Toxicity was not significantly different between thalidomide and comparators except for grade 3/4 neuropathy, which was worse in the thalidomide arm (RR 5.57, 95%CI 2.37-13.1, P<0.001). The rate of thromboembolic events (TE) grade 3/4 was reported in four trials. No significant difference was detected although there was a trend to more thromboembolic events in the thalidomide arm (RR 1.68, 95%CI 0.96-2.95, P=0.07). *Conclusion.* In our meta-analysis we found an improved PFS and for the first time also an improved OS for thalidomide maintenance therapy in patients with MM. This effect was accompanied by a higher rate of grade 3/4 neuropathy whereas the rate of TE was not increased.

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0943

THE ROLE OF THALIDOMIDE MAINTENANCE FOLLOWING HIGH DOSE THERAPY IN PATIENTS WITH MULTIPLE MYELOMA

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Background. Thalidomide has well established role in the therapy of multiple myeloma (MM) referring mainly to induction or consolidation therapy. Limitations of conventional chemotherapy initiated application of the high-dose treatment and autologous stem cell transplantation (ASCT) in MM patients (pts). The aim of study was to analyse results of the thalidomide maintenance following high dose therapy in MM pts. Patients and *Methods.* The study included 50 newly diagnosed MM pts (29 male/ 21 female, mean age 53 yrs, range 41-65). IgG myeloma was diagnosed in 28pts, IgA in 10pts, light chains in 10pts and non-secretory in 2pts. According to the clinical stage (CS, Durie-Salmon), patients were distributed as follows: II 19pts; III 31pts. Regarding ISS score, the group included: ISS1 8pts; ISS2 18pts; ISS3 24pts. Renal impairment was present in 3pts. Conventional induction treatment according to the VAD regimen (mean no. 4 cycles, range 3-6cycles) was applied in 35/50pts (70%). In 15/50 pts (30%) was applied induction treatment according to the CTD regimen (mean no. 4 cycles, range 3-6cycles). Stem cell mobilization was performed in all pts by combination of the CAD chemotherapy and G-CSF. High-dose therapy (HDT) with Melphalan 200 mg/m^2 and ASCT were performed 4-8 weeks after mobilisation. In the pts group treated with VAD induction, in 9pts who failed to achieve CR/VGPR (EBMT criteria), tandem ASCT was performed during average period 6m (range 3-12m) after first ASCT. In 10/35pts (27%) treated with VAD induction, Alfa-Interferon maintenance was applied after ASCT. Thalidomide maintenance (100 mg/day) was applied in 35/50 pts (70%) treated with VAD/CTD induction with median duration 19m (range 6-36 m). Routine thromboprophylaxis was applied in all pts receiving

thalidomide. *Results.* In the group of pts treated with VAD, VGPR was achieved in 4/35 pts (11%), and PR in 25/35pts (72%) after induction treatment. After HDT, CR was achieved in 7/35pts (20%); VGPR in 9/35pts (26%); and PR in 14/35pts (40%). Further improvement in response was obtained after tandem ASCT by achievement of VGPR in 8/9pts. In the group of pts treated with CTD, CR was achieved in 3/15pts; VGPR/PR in 8/15pts. Median follow-up was 26m (18-60m). The 3-yrs probability of event-free and relapse-free survival was significantly improved in the group treated with CTD induction and thalidomide maintenance (VAD+HDT+aIFN: EFS 20%, RFS 25% *vs.* VAD+HDT+Thal: EFS 30%, RFS 37% *vs.* CTD+HDT+Thal: EFS 45%, RFS 51%, P<0,0025). Furthermore, according to the analysis of 3-yrs probability, pts treated with thalidomide maintenance had a significantly longer overall survival (VAD+HDT+aIFN: 70% *vs.* VAD+HDT+Thal: 77% *vs.* CTD+HDT+Thal: 85%, P<0,0039). The main reason for thalidomide discontinuation was peripheral neuropathy recorded in 21/35pts (60%) with occurrence of grade 3-4 toxicity in 4/35pts. Thrombosis was not a risk in this setting. *Conclusion.* Thalidomide maintenance improves quality and duration of the response, as well as overall survival after HDT, mainly due to the better quality of response in the thalidomide group and further reduction of tumor mass after HDT. However, recommended duration of such treatment is to be defined predominantly due to the limitations caused by the toxic effects.

0944

A MULTICENTER, SINGLE-ARM, OPEN-LABEL SAFETY AND QUALITY OF LIFE STUDY OF LENALIDOMIDE PLUS DEXAMETHASONE IN PREVIOUSLY TREATED PATIENTS WITH MULTIPLE MYELOMA

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Background. Lenalidomide is an oral IMiD® immunomodulatory compound with established clinical efficacy and safety in patients with multiple myeloma (MM). Lenalidomide plus dexamethasone (Len+Dex) was well tolerated and demonstrated significant improvements in response and favorable overall survival compared with Placebo+Dex in two pivotal phase III registration trials in patients with relapsed/refractory MM (RRMM; Weber *et al.*, NEJM 2007; Dimopoulos *et al.*, NEJM 2007). *Aims.* In this phase III study, Len was administered to 587 patients with RRMM to assess the safety of Len+Dex and its impact on quality of life (QoL). *Methods.* Eligible patients with progressive disease (PD) after >2 cycles of anti-myeloma treatments, including thalidomide and bortezomib, or with PD having relapsed after treatment were enrolled into 3 geographic cohorts (Spain, UK/Ireland, and Austria/Australia). Patients received Len (25mg/day, day 1-21 every 28-day cycle) plus pulsed high-dose Dex (40mg/day, day 1-4, 9-12, and 17-20 every 28-day cycle for Cycles 1-4). Starting at Cycle 5, Dex was reduced to 40mg/day for days 1-4 every 28-day cycle. Deep vein thrombosis (DVT) prophylaxis with aspirin, warfarin, or low-molecular-weight heparin was strongly recommended. Bisphosphonates, erythropoietin, and antibiotics were administered at Investigator's discretion. The primary endpoint was safety of Len+Dex as measured by physician-reported adverse events (AEs; MedDRA 9.0 and NCI CTCAE 3.0). EORTC QLQ C-30 and QLQ MY-20 questionnaires at baseline and week 24 provided QoL outcome assessments. *Results.* 587 patients receiving ≥1 dose of Len+Dex were evaluated for safety and QoL. Median age was 65 years (273 [46.5%] were >65 years). During the study, 116 (19.8%) patients had ≥1 dose reduction due to treatment-emergent AEs. Prior to enrollment, 437 (74.4%) patients had received thalidomide, 209 (35.6%) had received bortezomib, 77 (13.1%) patients had a history of DVT, and 350 (59.6%) had a history of peripheral neuropathy. AEs observed in this study were consistent to those previously reported with Len+Dex. Grade 3/4 hematologic events were experienced by 267 (45.5%) patients, including neutropenia in 207 (35.2%) patients (Grade 3:167 [28.4%], Grade 4: 40 [6.8%]), thrombocytopenia in 89 (15.2%), anemia in 80 (13.6%), and febrile neutropenia in only 21 (3.6%). DVT (all grades) was experienced in 37 (6.3%), and new-onset peripheral neuropathy in 29 (4.9%) (Grade 3: only 1 [0.2%], no Grade 4). EORTC QLQ-30 revealed no significant median change (>5 points) from baseline in 14 of 15 scales for patients completing questionnaires at baseline and 24 weeks. Median fatigue increased in the UK/Ireland population (score 11.1). EORTC QLQ MY-20 revealed no significant median change from baseline of all scores except a statistically significant improvement in future perspective in Spanish patients (median 11.1), for patients completing questionnaires at baseline and 24 weeks. *Conclusions.* Len+Dex has previously been reported to significantly improve response and demonstrated favorable overall survival in patients with RRMM compared to Placebo+Dex. Despite co-treatment with high-dose Dex, preexisting neuropathy in >50% patients, prior MM treatment, and late disease course, patients were able to maintain median QoL scores over 24 weeks. High tolerability of Len+Dex in RRMM was also confirmed. (Data update at meeting).

0945

THE COMBINATION OF LENALIDOMIDE WITH LOW DOSE DEXAMETHASONE AND CYCLOPHOSPHAMIDE IS AN EFFECTIVE REGIMEN FOR PATIENTS WITH PRIMARY SYSTEMIC (AL) AMYLOIDOSIS: RESULTS OF A PHASE 1/2 STUDY

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Background. Lenalidomide (R) and cyclophosphamide (C) have shown activity in primary systemic (AL) amyloidosis. We designed a phase I/II trial of the combination RdC, with low-dose dexamethasone (d), in AL. *Aims.* Primary objective was to determine the MTD for RdC and to assess hematologic response. *Patients/Methods.* Patients received dexamethasone 20 mg on days 1-4, cyclophosphamide on days 1-10, lenalidomide on days 1-21 every 28 days, according to dose level (level 0: R 10 mg, C 50 mg; level 1: R 10 mg, C 100 mg; level 2: R 15 mg, C 100 mg). In the phase I part of the study, patients were observed for 2 cycles for determination of DLT, according to a standard 3+3 design. Patients with a creatinine ≤2.5 mg/dL and adequate hepatic function were enrolled. All patients received anticoagulation. *Results.* So far, 31 patients have been enrolled in the study (16 in phase I, 15 in phase II). In the phase I study, 3 patients were enrolled in dose level 0, 7 in dose level 1 and 6 in dose level 2. Dose level 2 is being further explored; 15 patients have been enrolled in phase II and accrual is ongoing. So far, 12 patients have received ≥6 cycles and 8 have completed 12 cycles. Heart was involved in 62% of patients, kidneys in 62%, liver in 12% and 19% had AL-related peripheral neuropathy; 95% of patients were Mayo stage II or III and 65% were previously untreated. Hematologic response rate, for patients who received ≥2 cycles (n=26), was 58% for all cohorts and 63% for patients treated at dose level 2. Median time to hematologic response was 2.9 months for all patients and 1.4 months for those treated at dose level 2. Organ responses have been recorded in 5 patients so far. After a median follow-up of 10 months, 14 patients have died (9 due to progressive heart amyloid). The most common hematologic toxicities were anemia (grade 3/4 in 17%) and neutropenia (grade 3/4 in 19%). Most common non-hematologic toxicities included infection (grade ≥3 in 22%), fatigue (grade ≥3 in 9%), and rash (in 22%, grade 3 in 4%). In parallel, and on a compassionate basis, AL patients with creatinine >2.5 mg/dL or on dialysis, were offered RdC with lenalidomide dose adjusted according to CrCl. Initially lenalidomide was given 15 mg every other day for CrCl <30 ml/min or 15 mg thrice per week on the day after dialysis, but due to toxicity, lenalidomide dose was reduced to 10 mg. So far, 13 patients have been treated with attenuated-dose RdC: 3 patients achieved a CR, one achieved a cardiac and one a liver response. The non-hematologic toxicity was significant including fatigue (53%), infections (38%), rash (31%), diarrhea (15%) and hyponatriemia (15%). Three patients discontinued treatment due to toxicity after the first cycle. *Summary/Conclusions.* RdC is an effective oral regimen for patients with AL amyloidosis. Toxicity is manageable for patients with serum creatinine <2.5 mg/dL but can be significant for patients with more severe renal impairment.

0946

OUTCOME OF AUTOLOGOUS STEM CELL TRANSPLANTATION FOR AL-AMYLOIDOSIS IN SWEDEN

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Background. High-dose melphalan with autologous stem cell transplantation (HDM/ASCT) is considered to be an effective therapy in AL-amyloidosis, but with high treatment related mortality. In Sweden, patients with AL-amyloidosis have been treated with HDM/ASCT since 1994, and no report has been made on the outcome. *Aims.* To evaluate all patients with AL-amyloidosis treated with HDM/ASCT in Sweden regarding response, survival and treatment related mortality. *Methods.* Information was collected on all patients treated at the six centra in Sweden where HDM/ASCT was performed. Only patients with amyloid classified as AL were included. Patients with myeloma or lymfoma, and those with only soft tissue involvement were excluded. Criteria from the 10th International Symposium on Amyloid and Amyloidosis (2005) were used to establish organ involvement, response and progression. *Results.* 67 patients treated with HDM/ASCT between 1994 and 2009 were evaluated. Median age at time of treatment was 58 years (range 41-69). 29 patients (43%) had cardiac involvement, 52 (78%) had kidney involvement and 17 (25%) had liver involvement. In 5 patients (7.5%) all three of these organs were involved, in 24 patients (36%) two of the organs were involved and in 35 patients (52%) one of the organs was involved. 3 patients (4.5%) had only gastrointestinal involvement. Response, either organ or haematologic, was seen in 36 patients (54%). Median time to response was 4.5 months (range 1-24). Of all 67 patients, 41 patients (61%) were alive at time of evaluation. Median follow up was 49 months (range 1-176). For the whole patient group overall median survival was not reached. Whereas for patients with cardiac involvement median survival was 28 months. Mortality within 100 days from ASCT was 13.4%. Causes of death were sudden cardiac death (n=3), heart failure (n=3), sepsis (n=2) and liver failure (n=1). *Conclusions.* HDM/ASCT leads to response and prolonged survival to a substantial degree. For patients with cardiac involvement survival is worse. Treatment related mortality is high (13.4%), but on a level with what is seen in other studies.

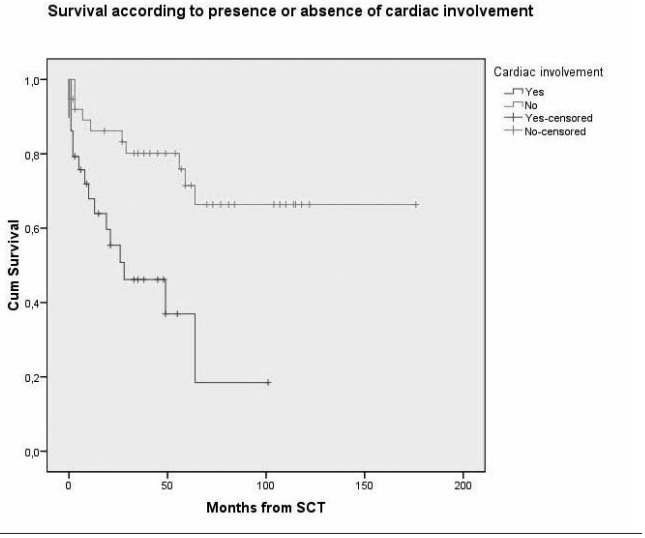

Figure.

0947

IN VIVO VISUALIZATION OF AL- AMYLOID DEPOSITS IN THE HEART USING [11C]PIB AND [11C]ACETATE AND POSITRON EMISSION TOMOGRAPHY (PET)

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Background. SAP scintigraphy is used for visualizing systemic AL amyloidosis, however the method is not reliable regarding amyloid deposits in the heart. *Aims.* To develop a method for visualizing amyolid deposition in the myocardium. *Methods.* After informed consent was obtained, 7 patients with AL amyloidosis affecting the heart and 2 healthy volunteers were examined with PET using tracers [11C]PIB and [11C]acetate. PIB (Pittsburgh Compound B) binds to amyloid. When combined with 11C, [11C]PIB is a PET amyloid imaging agent, whereas [11C]acetate measure blood flow in the heart. The amyloid type AL was confirmed with immunochemistry. Heart involvement was diagnosed either directly by myocardial biopsy (n=2), or indirectly by identification of AL amyloid in biopsy from another location in combination with UCG findings consistent with amyloid deposition. *Results.* All patients showed [11C]PIB retention in the heart whereas the uptake in healthy volunteers followed blood kinetics without any sign of retention. Our data thus indicates that we can visualize amyloid deposits in the myocardium of patients diagnosed with systemic AL amyloidosis measured as uptake and retention of the radiotracer [11C]PIB. [11C]PIB and [11C]acetate had different uptake patterns in patients which shows that [11C]PIB uptake is not representing blood flow and probably is a measure of binding to amyloid. Preliminary evaluation of the data show different patterns and degree of [11C]PIB uptake, which tentatively could be interpreted as heterogeneous amyloid deposits in the heart. We attempt to develop a tracer kinetic model, based on the assumption that the [11C]PIB uptake represents binding to amyloid, to quantify the amounts of amyloid deposits in the myocardium which could be correlated with the severity of the disease. *Conclusions.* The use of [11C]PIB and PET could be a method to study the distribution of deposits in systemic amyloidosis affecting the heart and most importantly, a non-invasive method for treatment follow-up.

0948

AN ANALYSIS OF THE ENDOCRINE DISEASE ASSOCIATED WITH SYSTEMIC AL AMYLOIDOSIS

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Background. Amyloid infiltration of the thyroid and testes has been previously reported but hormone function has not been studied in detail. *Aims.* We analysed the hormone profile of patients with systemic AL amyloidosis and correlated this with symptoms, extent of amyloidosis, and overall survival. *Methods.* All patients attending the National Amyloidosis Centre, UK with newly-diagnosed systemic AL amyloidosis between December 2008 and April 2009 were studied. Patients were tested for free T4 (FT4), thyroid stimulating hormone (TSH), luteinizing hormone (LH), follicule stimulating hormone (FSH), testosterone and sex hormone binding globulin (SHBG). They completed questionnaires for symptoms of hypothyroidism, date of menopause and erectile dysfunction. Analysis of amyloidotic organ involvement and function and medications potentially influencing hormone measurements was undertaken. *Results.* 79 patients (44 men, 35 women) were included, aged between 38 - 84 years (median 65 years). Cardiac involvement and autonomic neuropathy was observed in 50 (63%) and 21 patients (27%) respectively. Proteinuria >1g daily was seen in 66%, serum albumin <30 g/L in 30% and eGFR <30 mL/min in 19%. Symptoms of hypothyroidism were common: 62 patients complained of fatigue; 33 of excessive cold; 23 of constipation; 23 of deepening voice; 20 of low mood; and 8 of weight gain. 32 men (73%) had decreased or absent erectile function, 21 with autonomic neuropathy, low testos-

terone or both. All women were post-menopausal with no evidence of disease-related early menopause (all >51 years). Median TSH was 2.98 mU/L (range 0.19 - 15.95). A high TSH was observed in 19 patients (12 men, 7 women); only 2 had a low FT4 also (biochemically hypothyroid) with the other 17 patients having subclinical hypothyroidism. Eight were on medications that can confound measurements. Seven patients (2 men, 5 women) were on thyroxine. The median number of amyloidotic organs for those with high TSH and/or on thyroxine was 3 (range 1-4) *vs.* 2 (range 1-6) for other patients. There was no difference in median symptom scores between those with abnormal thyroid measurements and/or on thyroxine and other patients. Median testosterone in men was 10.5nmol/L (range 2 - 24.8). 21 men (49%) had low testosterone, 13 with erectile dysfunction, 9 with elevated FSH and/or LH. FSH was below normal in 1 man and 6 women. LH was below normal in 5 women. Raised SHBG was seen in 9 women and 30 men - this did not correlate with proteinuria, albumin or testosterone levels. 24 patients have died: 9/21 with thyroid profile abnormalities and/or who were on thyroxine (43%) compared to 15/58 (26%) without either characteristic (P=0.24). There was no difference in survival between low and normal testosterone men. *Conclusions.* Symptoms of hormone dysfunction are common in AL amyloidosis and are not reliable for predicting biochemical abnormalities. Interpretation of endocrine tests is complex due to medications and organ impairment. A third of patients had abnormal thyroid measurements and/or were on thyroxine, possibly associated with larger amyloidotic disease bulk. Low testosterone and impotence is common. With improving outcomes in AL amyloidosis, further analysis, including the role of hormone replacement should be undertaken.

0949

PREVALENCE OF MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE IN A DENSELY POPULATED, HIGHLY INDUSTRIALIZED AREA IN GERMANY

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Background. We utilized the biobank of the ongoing population-based, prospective Heinz Nixdorf Recall study to determine the prevalence of monoclonal gammopathy of undetermined significance (MGUS) in the densely populated and highly industrialized Ruhr area in Germany. *Methods.* The Heinz Nixdorf Recall study cohort comprises 4814 men and women from 3 large adjacent cities. Subjects were randomly selected from statutory lists of residence and gave informed consent. We screened serum samples from the baseline examination which took place from 2000 until 2003. Standard serum electrophoresis was combined with parallel screening immunofixation electrophoresis (IFE) using pentavalent antisera (Hydragel 12 IF, Penta-Kit, Sebia, Fulda, Germany). Where a monoclonal band was visible or suspected, confirmatory IFE followed. All gels were evaluated independently by LE and AH. Definition of MGUS cases was based on common criteria including monoclonal protein concentration, laboratory results, and disease history. Direct standardization with U.S. population 2000 as reference was performed to compare results to those of Olmsted County, U.S., published by Kyle *et al*. To quantify the impact of the screening IFE in detecting monoclonal proteins, we re-evaluated standard electrophoresis strips using a mask covering the pentavalent tracks on the gels. *Results.* 165 MGUS cases were identified in a total of 4708 screened samples, translating into a prevalence of 3.5% (95% confidence interval, 3.0-4.1). The median age of MGUS cases was 63 years (range 47-75), 103 (62%) were of male gender, and we observed an increase in prevalence with increasing age. The age-standardized prevalence was 3.9% (95% CI 3.2-4.5) which was higher than that reported for Olmsted County (P<0.05). We found the following distribution of monoclonal protein isotypes: IgG 59%, IgA 17%, IgM 28%, biclonal 2.4%, kappa 55%, and lambda 44%. Concentrations of the monoclonal proteins ranged from unmeasurable - 22.4 g/L with a median of 5.3 g/l. After a median observational time of 5 years, 3 MGUS cases progressed to multiple myeloma and 1 case developed a diffuse large B-cell lymphoma, representing a progression rate of 0.5%/year (95% CI 0.13 - 1.3). After re-evaluation of >1000 gel strips with masked pentavalent tracks, 9 MGUS samples previously identified were not detected. All but one had unmeasureable M-protein concentrations. Notably, the sample with a measurable M-protein was identified as IgA and the protein was hidden within the physiologic beta fraction. *Conclusion.* The higher age-standardized prevalence of 3.9% in the Heinz Nixdorf Recall cohort compared with that reported by Kyle *et al*. and the differences in isotype distribution (IgG 59% *vs.* 69%, IgA 17% *vs.* 11%) may at least in part be explained by the different screening strategies. Our preliminary results suggest that screening IFE increases sensitivity to detect monoclonal proteins and may be especially helpful in detecting IgA monoclonal proteins. A complete analysis of the gel re-evaluation using the cover for the pentavalent tracks will be presented at the conference. Whether environmental factors might also contribute to the increase in prevalence is the focus of ongoing research.

0950

LENALIDOMIDE PLUS LOW DOSE DEXAMETHASONE AS FIRST LINE THERAPY IN PATIENTS WITH PRIMARY PLASMA CELL LEUKEMIA: PLANNED INTERIM ANALYSIS OF A PILOT STUDY FROM THE GIMEMA-ITALIAN MYELOMA NETWORK

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Background. Primary Plasma Cell Leukemia (PPCL) is an aggressive, rare variant of myeloma characterized by poor prognosis . Lenalidomide is an IMiD immunomodulatory agent with proven efficacy in myeloma. No data are currently available on the use of lenalidomide as first line therapy in PPCL. *Aims.* On March, 2009, we started an open-label, multicenter, exploratory, single-arm, two-stage study aiming to evaluate safety and antitumor activity of the lenalidomide/low dose dexamethasone combination (Rd) in previously untreated PPCL. The primary endpoint was response rate according to International Uniform Criteria. The secondary endpoints were TTP, PFS, OS, percentage of eligible patients able to undergo stem cell transplantation (SCT) after Rd, and safety. *Methods.* Newly diagnosed patients with PPCL receive lenalidomide at a dose of 25 mg daily for 21 days of each 28-day cycle. Oral dexamethasone is administered at a dose of 40 mg daily on days 1, 8, 15, and 22 for each 28-day cycle. After 4 cycles, patients achieving at least PR and not eligible for SCT continue until 8 cycles of full-dose Rd, if tolerated, followed by a maintenance dose of single agent lenalidomide equal to 10 mg/day on days 1-21 of each 28-day cycle. Patients responding after 4 Rd cycles and eligible for SCT proceed according to single Centre transplant policy. Patients not responding after 4 cycles or progressing during Rd treatment are considered off-study. Appropriate dose reductions, contraception methods and anti-thrombotic prophylaxis are required. *Results.* According to the Simon Optimal Two-Stage Adaptive Design, a planned interim analysis was done (Stage 1) to evaluate the first 12 enrolled patients (5 male, 7 female, median age 64, range 45-81), out of 22 total patients required to complete Stage 2 of the trial. At baseline, circulating plasma cells ranged from 655 to 34,000 × 10e9/L. Six patients had a moderate degree of renal failure (serum creatinine levels up to 2.6 mg/dL), five had increased LDH, three had extramedullary disease, nine had anemia and/or thrombocytopenia. Among 11 patients with cytogenetic analysis, nine had del13: two in combination with del17 and t(14;16), two with t(14;16), one with del17 and t(11;14), one with del17, one with t(4;14). One patient had del17 alone. After a median of 3 Rd cycles (range 1-5), 1 CR, 1 nCR, 3 VGPR, and 4 PR were achieved (ORR 75%). Two of responders underwent adequate peripheral blood stem cell collection followed by autologous SCT. One patient died during the first cycle because of progressive disease. Two patients developed extramedullary disease under Rd therapy, despite the complete clearance of circulating plasma cells. One of responders died in PR, due to causes unrelated to PPCL or treatment. Grade 3-4 hematological toxicities occurred in 8 patients. Two patients experienced pneumonia, one patient an intestinal perforation due to fecalith. After a median follow-up of 7 months, ten patients are alive (83.3%), eight of whom still responders. *Conclusions.* Rd is a promising initial therapy for PPCL patients. An update of this study, including SNP and GEP analyses, will be presented at the Meeting.

0951

LONG, EVEN EXCEEDING 20 YEARS, SURVIVAL IN MULTIPLE MYELOMA

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The study reports the long-term survival of 600 multiple myeloma patients on conventional chemotherapy, with a follow-up of at least 10 years. The study was especially focused on estimation of real frequency of long-term survivals in patients with multiple myeloma and finding common clinical and laboratory features present in long-term surviving patients as possible good prognostic factors. Of 600 studied patients with multiple myeloma diagnosed before 2000 and treated in our Institute 88 (14.7%) survived over 7 years, including 45 (7.5%) over 10 years, 11 (1.8 %) over 15 years and 7 patients (1.1%) over 20 years (Table 1) from the disease diagnosis and beginning of antitumor treatment. The patients with long survival were younger (median age 55 years) at diagnosis than those in a whole studied group and had normal creatinine, calcium and beta2-microglobulin levels in the serum. Sixty eight percent of these patients presented I or II stage of clinical progression, 60% had IgG monoclonal protein and 58% showed osteolysis. Treatment with melphalan only, was given to 18 patients, 30 were treated with melphalan followed by vincristine, cyclophosphamide, BCNU, doxorubicin and prednisone or dexamethasone. Beginning from the time of diagnosis, polychemotherapy was given to 16 patients, 15 patients, besides chemotherapy, received radiotherapy or 60Co irradiation and 9 received new agents: thalidomide, bortezomib, lenalidomide. In 66% of evaluated cases the response to treatment was good and in remaining 34% of cases stabilization of the proliferative process was achieved. The mean duration of the treatment till achieving of partial response was 10 months, ranging from 2 to 89 months. The mean duration of the good therapeutic response was 70 months. Twelve patients are alive and further treated and 7 patients remain without treatment. The longest follow- up of a still living patient with multiple myeloma is 31 years after detection of monoclonal protein and 25 years after beginning of antitumor treatment. The longest follow-up of a still living patient with initially isolated bone involvement is 23 years after detection of the first bone lesion and 19 years, after generalization of the process. In 6 cases, the cause of death was acute myeloid leukemia in 5 cases solid tumors. In 2 patients with myeloid leukemia no plasma cell infiltrates were found at autopsy; it confirms curing multiple myeloma.

Table 1. Parients with myeloma surviving ≥ 20 years.

Case	Sex	Age	M-protein	Osteolysis	Main therapy	Survival years	Cause of death
1.	F	40	IgAκ	-→+	MP	33	myeloma progression
2.	F	53	IgGκ	-→+	M, Rtx	>25	
3.	M	40	IgGκ	+	Rtx, MP, VMCP	>23	
4.	F	52	IgGκ	-→+	VMBCP	22	myeloma progression
5.	F	41	0→BJκ	-→+	Surgical Rtx, M, VBCP	22	myeloma progression
6.	M	43	IgGκ	-	M, VBCP	20	myeloma progression
7.	F	57	BJλ	+	MP, VMCP	20	myeloid leukemia

Abbreviations: B- BCNU; C- cyclophosphamide; M-melphalan; P- prednisone, V-vincristine; Rtx-radiotherapy.

0952

BORTEZOMIB-CONTAINING COMBINATIONS AS FRONT-LINE THERAPY IN PRIMARY PLASMA CELL LEUKEMIA: A RETROSPECTIVE STUDY FROM GIMEMA MULTIPLE MYELOMA AND ACUTE LEUKEMIA WORKING PARTIES

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Background and Aim. The aim of this study was to evaluate the role of chemotherapy combinations including bortezomib as first line therapy in primary plasma cell leukemia (PPCL), a rare variant of multiple myeloma, characterized by very poor prognosis. To this purpose, a retrospective survey was performed in 21 hematologic Italian Institutions of previously untreated patients with PPCL who had received bortezomib associated with other agents for the initial treatment of their disease. *Methods.* Twenty-nine unselected and previously untreated PPCL patients were collected (M/F ratio 1.3; mean age 62 years, range 47-82). Circulating plasma cells ranged from 10 to 95% (mean 39%). Median WBC count was 14.6 x 10e9/L (range 2.2-81). Variable degrees of renal impairment were observed in 14 patients. Seven patients had concomitant extramedullary disease. Cytogenetic/FISH abnormalities were observed in 14 out of 17 evaluated patients, the most frequent being complex caryotype (including various combinations of t(4;14), t(14;16), del17 and del13q. Bortezomib was given using the standard schedule of 1.3 mg/sqm days 1, 4, 8, 11, with an interval of 10 days between cycles. Nine patients received bortezomib in combination with dexamethasone and thalidomide (VTD), seven with dexamethasone alone (BD), seven with doxorubicin and dexamethasone (PAD), two with oral melphalan and prednisone (VMP), two with doxorubicin, dexamethasone and vincristine (PAD-V), one with melphalan, prednisone and thalidomide (VMPT) and one with cyclophosphamide and dexamethasone (BCD). A total number of 104 cycles was administered (mean 3.7, range 1-9). After bortezomib containing induction therapy, seven patients underwent double (n.4) or single (n.3) autologous stem cell transplantation (AuSCT), 4 patients underwent AuSCT followed by reduced intensity (RIC) allogeneic stem cell transplantation (Allo-SCT, one with an unrelated donor), while one patient underwent myeloablative Allo-SCT. *Results.* According to the International Uniform Response Criteria, twelve partial remissions, three very good partial remissions, and eight complete remissions were achieved (overall response rate: 79.3%). After a median follow-up of 24 months, sixteen patients are alive (55%): twelve out of them remain in remission phase, four relapsed after 4, 11, 16 and 31 months, respectively. Eleven out of the 13 deceased patients did not receive stem cell transplantation. Grade 3-4 hematological, neurological, infectious and renal toxicities occurred in 5, 6, 4 and 1 patients, respectively. *Conclusions.* These findings confirm and extend previous data suggesting that chemotherapy combinations including bortezomib are safe and effective first line treatments for PPCL, in particular in patients eligible for stem cell transplantation.

0953

SERUM IMMUNOGLOBULIN HEAVY/LIGHT CHAIN RATIOS ARE INDEPENDENT RISK FACTORS FOR PREDICTING PROGRESSION FREE SURVIVAL IN MULTIPLE MYELOMA

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Background. Genetic aberrations have emerged as important prognostic factors in multiple myeloma (MM), although detection methods are not readily available and require skilled personnel. The current international staging system (ISS) for MM relies upon measurement of Beta 2 Macroglobulin (β2M) and albumin. Measurement of immunoglobulin production is not prognostic, but is required for monitoring. Newly developed nephelometric assays allow the accurate measurement of serum heavy/light chain (IgAκ / IgAλ and IgGκ / IgGλ) proteins; the measurement of which can quantify the immunoglobulin produced by the tumour and indicate the level of polyclonal immunoglobulin suppression. *Aims.* To determine the prognostic value of serum immunoglobulin heavy/light chain (HLC) ratios. Furthermore, to comment on the role of isotype specific *vs.* systemic immunoparesis in MM. *Methods.* Archived, presentation sera from 339 patients enrolled on the IFM 2005-01 trial were assayed. Free light chain (FLC), β2M and albumin were measured in all sera. In addition, IgGκ & IgGλ concentrations were measured in sera from the 245 IgG MM patients (166 IgGκ, 79 IgGλ). IgAκ and IgAλ concentrations were measured in the sera from the 94 IgA MM patients (60 IgAκ, 34 IgAλ). Association with progression free survival (PFS) was determined using univariate and multivariate analysis (SPSS Version 18). *Results.* Cox regression analysis identified abnormal the HLC ratio as being associated with reduced PFS (P<0.001). The association was independent of other serum markers including B2M, albumin and markers of genetic aberrations including Del_13, t4_14 and Del_17p. There was good correlation between an increasing-

ly abnormal HLC ratio and the relative risk of progression (Figure 1). Separation of the HLC values into monoclonal protein production and polyclonal suppression clearly showed that monoclonal production was not associated with shorter PFS (P=0.142), similarly to SPE densitometry results (P=0.1). Levels of polyclonal isotype-matched immunoglobulin suppression were associated with shorter PFS (P=0.002), although not as significant as the overall HLC ratio (P=0.00005). Systemic immunoparesis (i.e. IgG and IgM in IgA MM and IgA and IgM in IgG MM) was defined as a reduction in immunoglobulin measurement ~33% below the normal range (IgG=5g/L, IgA=0.5g/L and IgM=0.3g/L). Levels of IgG and IgM were not prognostic in IgA MM (P=0.169, P=0.477), similarly level of IgA and IgM were not prognostic in IgG MM (P=0.952, P=0.977). *Summary.* Increasingly abnormal HLC ratios were associated with shortened PFS in MM patients. The association is independent of serum markers such as β2M, albumin, SPE densitometry and genetic markers (Del_13, t4_14 and Del_17p). Isotype matched polyclonal immunoglobulin measurements are significantly associated with shorter PFS, although the use of ratio is more powerful prognostically. Systemic immunoparesis is not associated with shortened PFS, supporting the presence of immunoglobulin specific myeloma niches in the bone marrow.

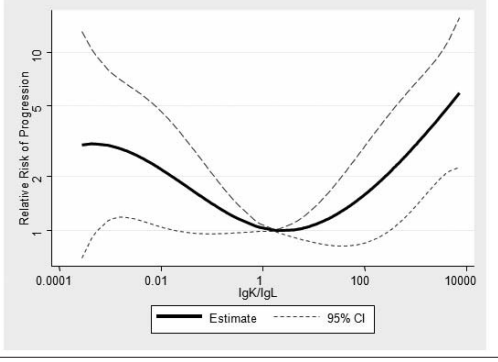

Figure 1. Relative risk of progression for heavy/l.

0954

CIRCULATING PLASMA CELLS (CPC) PREDICT THE OUTCOME OF RELAPSED OR REFRACTORY MULTIPLE MYELOMA (RR MM)

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Background. Pretreatment detection of peripheral blood malignant CPCs by immuophenotyping has been shown to be of negative prognostic value in MM and related disorders. The number of CPCs tends to decrease in response to treatment. We hypothesized that assessment of CPC kinetics in response to one therapy cycle may be of prognostic significance and could be helpful in the early detection of MM refractoriness to treatment. *Aims.* The aims of our study were to assess the prognostic significance of pretreatment values of normal and aberrant CPC subsets as well as the prognostic significance of CPC kinetics in response to the first treatment cycle in RR MM. *Methods.* Patients were prospectively included if they had RR MM according to EBMT criteria after at least one prior line of therapy and were scheduled to receive either a Bortezomib containing regimen or VAD (vincristin, doxorubicin and dexamethasone). All patients provided informed consent. We used six-color flow cytometry to identify immunophenotypically normal (nCPC) and aberrant plasma cell (aCPC) subsets in peripheral blood (PB). Assays were performed with two tubes stained with antibody combinations: CD56/CD138/CD45/CD19/CD38/CD20 and cLambda/cKappa/CD138/CD19/CD38/CD45. Plasma cells were identified as normal (nCPCs) if they were $CD138^+/CD38^+/CD19^+/CD56^-$/normal kappa/lambda ratio/CD45 variable or aberrant(aCPCs) if they displayed $CD138^+/CD38^+/CD19^-/CD56^{+/-}$/abnormal kappa/lambda ratio/CD45 variable. We measured aCPC and nCPC subsets immediately before and then after one therapy cycle in RR MM patients. *Results.* 31 adult patient was enrolled into the study. Median age was 59 years. After the median observation time of 16.7 months, patients with detectable pretreatment aCPCs had shorter time to progression (TTP) compared to patients with undetectable aCPCs (median 258 *vs.* 456 days, respectively (P=0.022)). TTP of 57 days and overall survival (OS) of 139 days was shortest in patients whose aCPCs increased after one therapy cycle compared to patients with decreasing (TTP 259 days and OS not reached) or undetectable (TTP and OS not reached) aCPCs (P<0.001 and P=0.043 for TTP and OS, respectively) (Figure 1). Detection of nCPC before treatment did not showed prognostic significance, however kinetics of nCPC were prognostically important: patients with either absent or decreasing nCPCs after one therapy cycle had significantly shorter TTP as compared to patients with increasing nCPCs (217 and 388 days, respectively (P=0.029)). *Summary.* Detection of aCPCs in PB before treatment may identify patients with more aggressive disease. Nonreduction of aCPCs in RR MM patients after the first cycle of therapy may be useful in identifying patients early who are resistant to the administered therapy. If our findings are confirmed in larger studies, these patients may be candidates for immediate switch to alternative therapy. Figure 1 Time to tumor progression in three aCPC kinetic groups: group I - patients with no detectable aCPCs in both pre and postchemotherapy samples, group II - patients with a decrease in aCPCs postchemotherapy as compared to aCPCs before chemotherapy, group III - patients with no change or increase in aCPCs postchemotherapy as compared to aCPCs before chemotherapy.

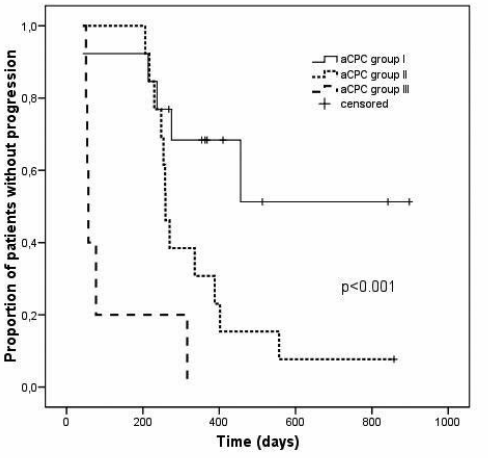

Figure 1.

0955

PREVALENCE OF MONOCLONAL GAMMOPATHY OF UNKNOWN SIGNIFICANCE IN KOREAN URBAN ELDERLY POPULATION

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Background. Studies on the epidemiology of monoclonal gammopathy of undetermined significance (MGUS), a premalignant precursor of myeloma, are limited in Korean. *Aims.* The aim of this study is to investigate the prevalence and characteristics of MGUS in an elderly urban Korean population. *Methods.* Among 1,000 randomly sampled participants aged more than 65 years (sourced from the Korean Longitudinal Study on Health and Ageing in 2006), 945 subjects were evaluated for MGUS. MGUS was assessed using agarose-gel electrophoresis (SPIFE 3000, Helena), immunofixation (SPIFE 3000, Helena) and free light chain quantitation (BNII nephelometry, FREELITE The Binding Site Ltd.). Laboratory and radiologic tests were also performed. *Results.* MGUS was identified in 35 (3.7%) of the 945 study participants. The prevalence of MGUS was 1.7% among persons 65-69 years, 3.4% among 70-79 years and 5.8% among those 80 years or older. The prevalence increased with age and was higher in men (5.3%) than women (2.5%). The isotype of the M protein was IgA in 37%, IgG 29%, light chain 20% and IgM 14%. *Conclusions.* The prevalence of MGUS is slightly lower in this Korean population than that reported in Minnesota study among people older than 70 years (3.4% *vs.* 4.6%), but simi-

lar with Japanese data (3.4% *vs.* 3.0%). We suggest that MGUS is less prevalent among Korean elders than American. This is the first study to estimate the prevalence rate of MGUS in Korean elderly population and these findings are needed to confirm with further study using 2010's follow-up samples. We will also seek the transformed cases to multiple myeloma.

0956

WITHDRAWN BY AUTHOR

0957

COMPARISON OF FIXED DOSE PEGFILGRASTIM AND DAILY FILGRASTIM AFTER AUTOLOGOUS STEM CELL TRANSPLANTATION IN PATIENTS WITH MULTIPLE MYELOMA AUTOGRAFTED ON A OUTPATIENT BASIS

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Background. There is a growing demand for autologous stem cell transplantation (ASCT) in patients with multiple myeloma (MM), with increasing pressure on available hospital beds. Accordingly, different authors have explored the feasibility of autografting patients on an outpatient basis. G-CSF is used to accelerate hematopoietic recovery following ASCT, although there is no consensus about its optimal use in this setting. Pegfilgrastim (PEG), a long-acting recombinant G-CSF, has been shown to have similar efficacy when compared to conventional G-CSF for chemotherapy-induced neutropenia, but little is known about its use in the ASCT setting, namely in patients programmed to be autografted on outpatient basis. *Aims.* To compare therapeutic results in terms of hematopoietic recovery, non hematologic toxicity, duration of hospitalization and percentage of hospital readmission between patients receiving either conventional G-CSF or PEG. *Methods.* Forty-eight patients with MM were autografted by using PEG, given at a single dose of 6 mg at day +5 from stem cell infusion, while 113 received G-CSF from day + 2 up to stable neutrophil recovery (median 8 days, range 6-11). The conditioning regimen was high dose melphalan (HDM) in all patients (200 mg/sqm up to 65 years and 140 mg/sqm over 65 years). All patients were discharged the day after stem cell infusion as programmed. The median age was comparable between the two groups (57 for G-CSF and 59 for PEG, p:0.11). The median number of CD34+cell infused was 5.9×10^6/kg and was comparable in the two groups (5.8 for PEG and 5.9 for G-CSF, p:0.67). *Results.* Out of 161 patients, 125 (68%) did spend the aplastic phase entirely at home following HDM and stem cell infusion. A second hospital admission was required in 36 patients (32%). Febrile neutropenia and severe mucositis needing total parenteral nutrition were the most frequent causes of hospitalization. However, there was only one documented infection and either fever or mucositis were easily resolved at the time of hematopoietic recovery in all patients. Median time to neutrophil recovery for the whole patient population was 12 days and there was no difference between the PEG subgroup (12 days) and controls (12 days), P:0.87. In addition, the incidence of FUO (34% *vs.* 29 %, P:0.22) and grade 3 mucositis (18% *vs.* 20 %, p:0.34) was comparable. Finally there was no statistically significant difference as percentage of hospital readmission is concerned: in the PEG group 6 out of 48 patients (12%) were hospitalized as opposed to 30 out of 113 (26%), p:0.06. The median time of hospital stay for readmitted patients was 5 days (4-26) and was identical for the two subgroups (5 days *vs.* 5 days, p.0.94). Finally no case of transplant related mortality occurred in the whole patient series. *Conclusions.* ASCT on an outpatient basis is feasible and safe in patients with MM, the majority of whom are manageable at home. The administration of single dose PEG results in no different outcome in terms of safety and efficacy as compared to 8 days of G-CSF and is better accepted by patients.

0958

THE COMBINATION OF LENALIDOMIDE AND DEXAMETHASONE REDUCES BONE RESORPTION IN RESPONDING PATIENTS WITH RELAPSED/REFRACTORY MULTIPLE MYELOMA BUT HAS NO EFFECT ON BONE FORMATION

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Background. Lenalidomide plus dexamethasone (RD) is very effective for the management of refractory and relapsed multiple myeloma (MM). However, there is very little information for its effect on myeloma bone disease. *Aim.* The aim of this prospective study of the Greek Myeloma Study Group was to evaluate the effect of RD regimen on bone remodeling in relapsed/refractory MM. *Patients/Methods.* We evaluated 106 consecutive patients (54M/52F; median age 68 years) who received lenalidomide at the standard dose of 25mg PO daily (or adjusted to creatinine clearance) on days 1-21 of a 28-day cycle in combination with dexamethasone at a dose of 40mg PO on days 1- 4 and 15-18 for the first four cycles and only on days 1-4 thereafter. All patients were under zoledronic acid both pre- and during treatment period. The following serum indices of bone metabolism were measured on cycle 1/day 1, and then on day 28 of cycles 3 and 6: (i) osteoblast inhibitor dickkopf-1 (Dkk-1); (ii) osteoclast regulators: sRANKL and osteoprotegerin (OPG); (iii) bone resorption markers: CTX and TRACP-5b; and (iv) bone formation markers: bone-specific alkaline phosphatase (bALP) and osteocalcin (OC). These markers were also evaluated in 44 healthy controls of similar gender and age. *Results.* At baseline, 16 patients had no lytic lesions (14.8%, group A), while 35 (33%) had 1-3 lytic lesions (group B) and 55 (50.9%) had >3 lytic lesions and/or a pathological fracture (group C) in skeletal survey using conventional radiography. Patients at baseline had increased levels of Dkk-1, sRANKL, and bone resorption markers (P<0.01) and reduced levels of OC and bALP (P<0.01) compared to controls. Group C patients had increased Dkk-1 compared with all others (P=0.012). Patients with ostelolysis (groups B+C) had elevated levels of sRANKL/OPG ratio compared with group A (P=0.04). The pre-treatment values of sRANKL correlated with CTX and Dkk-1 (r=0.266, P=0.007 and r=0.200, P=0.047, respectively), while serum Dkk-1 correlated with TRACP-5b (r=0.403, P<0.001). To-date, 80 patients have completed 3 cycles of therapy while 55 have completed 6 cycles. The objective response was 55% (CR 12%, VGPR 11%, PR 32%). The administration of RD produced a reduction of Dkk-1 after 3 cycles of treatment in patients who showed an objective response to therapy (at least PR) but not in patients who did not respond to treatment (P=0.035). There was also a reduction in CTX serum levels (-4% median change) in responders (ranging from -100% to +209%) in contrast to patients who did not respond to RD and showed a median increase of 17% (ranging from -97% to +3913%; P=0.04). Non-responders had increased TRACP-5b serum levels after 3 cycles of treatment compared to baseline (P=0.04). There were no changes in markers of bone formation even in responders. *Summary/Conclusions.* RD regimen reduces bone resorption only in responding patients with relapsed/refractory myeloma but has no effect on bone formation. Combination with agents with known anabolic effect on bone, such as proteasome inhibitors or anti-Dkk1 drugs, may be of benefit for the management of bone disease in patients treated with RD.

0959

EXTRAMEDULLARY RELAPSED MULTIPLE MYELOMA HAS EXTREMELY POOR PROGNOSIS EVEN IN THE ERA OF THE NEW DRUGS

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Introduction. Multiple myeloma (MM) is 2nd most common hematological malignancy. Extramedullary lesions (EM) mean aggressive disease. Patients (pts) with EM have poor prognosis if conventional chemotherapy is used for treatment. About 10 % of MM patients eventually relapse with EM disease after autologous stem cell transplant. *Aims, methods.* The aim of this study was to define incidence and prognosis of patients with EM in era of new drugs. We studied all consecutive 226 relapsed MM pts (115M/111F,median age:61 years,median follow-up 3.7 years) who received treatment in our centre between 2005 and 2008. EM relapse was proven by CT or MRI if it was not possible to verify EM histologically. Pts with EM at the time of diagnosis were excluded from analysis. All pts were treated with thalidomide and/or bortezomib. Patients with EM were divided into two groups: first group with EM related to bone (B-EM) and second group pts with EM in soft tissues not related to bone or with plasmocyte infiltration of parenchymatous organs (ST -EM). Thalidomide (33%), bortezomib (38%) and lenalidomide (5%)-based regimens were used as treatment of EM relapse, in 42 % pts together with autologous transplantation. Mann-Whitney test and Log-rank test were used for evaluation of differences between groups of pts. *Results.* Totally 24% pts (55/226) had proven relapse with EM (42% (22/55) - B-EM; 58% (32/55) - ST-EM). The most common site of EM was skin infiltration

occuring in 42% (22/55) of pts. Surprisingly, EM was present in 53% (29/55) of pts in the first relapse, in 33% (18/55) of pts in second relapse but later only in 14% (8/55). Overall response rate was 24% (13/55) with 5% (3/55) of complete remission and 21% (10/55) partial remission. Time to progression in these pts was only 5.4months. Median of OS (89.5 months for all of 226 pts) was significantly shorter in the group with EM when compared to group without EM (109m *vs.* 38m; P=0.0001). Median OS in pts with ST-EM was also decreased compared to pts with B-EM (30mvs45m,P=0,002). Median OS from date of relapse was only 8m in all EM pts. However, OS in pts with ST-EM was shorter than in B-EM pts.(12mvs4m,P=0.006). *Conclusion.* Our data showed higher incidence of EM relapse in comparison with 90´s .Interval of survival after EM relapse and is extremely short if EM occurs, although all new drugs including IMIDs and bortezomib are used for the treatment. Our findings indicate that EM still means very poor prognosis of MM patients even in the era of the new effective drugs, especially if ST-EM occurs. We have not yet fully understood why the incidence of EM is so high in the last 4 years. In our opinion, the treatment of EM became a crucial problem in MM, and effective treatment is unfortunately not available.

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0960

MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE AND RISK OF VENOUS THROMBOEMBOLISM

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Background. The risk of venous thromboembolism (VTE) in multiple myeloma is increased but little information is available on the risk of VTE in patients with the precursor condition monoclonal gammopathy of undetermined significance (MGUS) and the results of previous studies are contradicting. *Aims.* To evaluate the risk of VTE in MGUS in a large population-based study. *Methods.* We identified 1,610 MGUS patients without prior VTE in the 1978-2006 period in North Jutland County, Denmark and 16,100 members of the general population were randomly selected from the Danish Central Population Registry, matched by age, gender, comorbidity and year of diagnosis. Data on VTE in the two groups were obtained from the National Patient Registry covering all Danish hospitals. The follow-up period began one year after detection of the M-component. Cox regression analysis was used to estimate the relative risk of VTE adjusting for age, gender and comorbidity. In addition, we used Cox-regression analysis to estimate the mortality rate ratio (MRR) of MGUS patientens with VTE compared to MGUS patients without VTE.

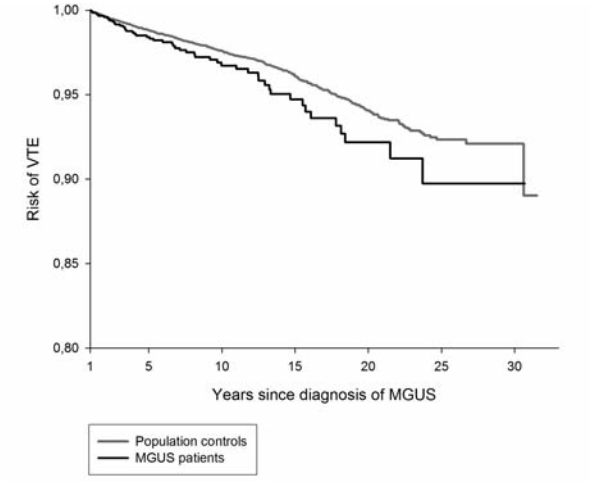

Figure 1.

Results. The MGUS cohort comprised 823 males and 787 females, all together with a median follow-up time of 6.0 years (range, 0-29.7 years), totaling 12,558 person-years at risk. The median age at MGUS was 70.1 years (range, 10.1-96.5 years). During follow-up 50 cases of VTE (28 cases of deep venous thrombosis of the legs and 22 cases of pulmonary embolism) were identified in the MGUS cohort, corresponding to an incidence rate of 4.0 VTEs/1000 person-years. The cumulative risks of VTE in MGUS patients and population controls are shown in Figure 1. The relative risk for VTE among MGUS patients compared to population controls was 1.37 (95% confidence interval (CI), 1.00-1.88). Only one MGUS patient with VTE was later diagnosed with malignant transformation. The crude MRR for MGUS patients with VTE compared to MGUS patients without VTE was 2.4 (95% confidence interval (CI), 1.7 - 3.4). After adjustment for age, sex, and comorbidity the MRR was 2.0 (1.4 - 2.8). *Summary/Conclusions.* The overall risk of VTE in the MGUS patients was increased compared to the population controls. The study does not support an association between VTE and later malignant transformation. Our results indicate that VTE in the MGUS patients is associated with reduced survival.

0961

COMPARE - RESULTS OF A RANDOMISED STUDY TO ASSESS THE RENAL SAFETY AND EFFICACY OF IBANDRONATE AND ZOLEDRONATE IN MULTIPLE MYELOMA PATIENTS

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Background. Bisphosphonates (BPs) are widely used to prevent skeletal complications in patients (pts) with Multiple Myeloma (MM), which is characterised by osteolytic bone lesions or diffuse bone disease. As MM pts are at high risk for renal damage, renal safety may be a discriminator for selecting a particular BP. *Methods.* COMPARE is a randomised, multi-centre, open-label, parallel-group study to assess the renal safety of ibandronate (IBA) and zoledronate (ZOL) in pts with MM stage II-III (Salmon, Durie). Exclusion criteria included prior treatment with IBA or ZOL, serum creatinine >4.0 mg/dL, and estimated creatinine clearance (eCrCl; Cockcroft-Gault equation) ≤30mL/min. Pts were randomised to receive IBA (6 mg) or ZOL (4mg), administered as IV infusion over 15 min Q4W for up to 92 weeks. ZOL dosage could be reduced at the physician's discretion. Primary endpoint was a relevant deterioration of renal function, defined as a decrease in eCrCl of ≥30% or to ≤30mL/min, respectively. Secondary endpoints included reductions of ZOL dose, number of, and time to first occurrence of, skeletal related events (SREs), and cases of osteonecrosis of the jaw (ONJ). Statistics were descriptive. *Results.* 81 pts were randomised to receive IBA (n=41) or ZOL (n=40). The number of pts experiencing a relevant decrease in renal function was similar for treatment with IBA (6 pts, 14.6%) and ZOL (5 pts, 12.5%), respectively; however, 12 pts (30%) in the ZOL group underwent at least one dose reduction. The mean treatment period was 52.6 and 44.3 weeks for IBA and ZOL treated pts, respectively (based on safety population). With IBA treatment, 9 pts (22.0%) experienced an SRE compared to 12 SREs (30.0%) reported in ZOL treated pts. Median time to first occurrence of an SRE was 393 days for IBA treatment and 244.5 days for ZOL treatment. ONJ was not observed in either treatment group. *Conclusions.* In this randomised, multi-centre, open-label, parallel-group study comparing the renal safety and efficacy of IBA and ZOL in pts with stage II-III MM, the incidence of relevant decreases in estimated creatinine clearance was similar between treatment groups. With 30% of ZOL treated pts having had at least one dose reduction, physicians may have titrated ZOL dosing to achieve acceptable renal safety. SREs seemed to occur earlier and more frequently with ZOL compared to IBA treatment.

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0962

ARTERIAL AND VENOUS THROMBOSIS IN PATIENTS WITH MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE: INCIDENCE AND RISK FACTORS IN A COHORT OF 1,491 PATIENTS

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Background. Monoclonal gammopathy of undetermined significance (MGUS) has been reported to be associated with an increased risk of venous thrombosis. *Aims.* To investigate the incidence of arterial and venous thrombosis in patients with MGUS and the associated risk factors. Patients and *Methods.* We carried out a retrospective multicenter cohort study on 1,491 patients with MGUS (M/F 789/702, median age at diagnosis 63 years, range 24-94). The type of M-protein was IgG in 1,115 patients (75.1%), IgA in 185 (10.4%), IgM in 173 (11.7%), and biclonal in 48 (3.2%); 520 (34.8%) had M-protein level >1.6 gr/L. Forty-nine (3.2%) of the patients were diagnosed as MGUS carriers at the time of a thrombotic event, with no significant difference in sex, age, type or level of M-protein in respect to the remaining patients. The patients without recent thrombosis (<2 years) when MGUS was diagnosed and with a recorded follow-up were 1,238; the risk of thrombosis during the follow-up was estimated as hazard ratio (HR) using a Cox model having as covariates gender, age >60 years at the time of diagnosis, history of remote thromboses (>2 years before diagnosis of MGUS), presence of cardiovascular risk factors, M-protein level >1.6 gr/L at diagnosis, and presence of biclonal gammopathy. *Results.* Thirty-three patients of 1,238 (2.6%) had thrombosis during the follow-up: nine acute coronary syndrome, nine cerebrovascular disease, one splenic infarction, seven deep venous thrombosis, five superficial vein thrombosis, and two retinal vein thrombosis. The total observation time was 7,334 years (median 3.3, range 1-27.7); thus, the overall incidence of thrombosis was 4.4 per 1,000 patient-years, 2.5 for arterial events and 1.9 for venous events. Multivariable analysis showed a significant increase in risk for arterial thrombosis in the patients having cardiovascular risk factors (HR 4.92, 95%CI 1.42-17.0), as expected; an increased risk for venous thrombosis was present in patients with M-protein level >1.6 gr/L (HR 3.34, 95%CI 1.06-10.48), and in patients with biclonal gammopathy (HR 9.02, 95%CI 1.07-75.61). No thrombotic event was observed in the patients who evolved to multiple myeloma (n=56) or another neoplastic disease (n=24). *Conclusions.* The incidence of arterial and venous thrombosis in the patients with MGUS does not seem higher than that expected in the general population. However, the risk of venous thrombosis is increased in the presence of M-protein >1.6 gr/L or biclonal gammopathy.

Myeloproliferative disorders - Biology 2

0963

THE C618R MUTATION OF JAK2 POTENTIATES THE JAK2V617F-INDUCED PHOSPHORYLATION AND ACTIVATION OF DOWNSTREAM TARGETS

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Background. A single *JAK2* exon 14 mutation (*JAK2*V617F) occurs in over 90% of patients with polycythemia vera (PV). Other *JAK2* exon 14 mutations have been recently described in association with *JAK2*V617F in PV patients. We previously reported the finding of a *JAK2*C618R alteration in a *JAK2*V617F-positive patient. Molecular studies revealed that *JAK2*C618R is also an acquired mutation and that *JAK2*V617F and *JAK2*C618R are located in the same *JAK2* allele. However, the functional consequences of *JAK2*C618R on *JAK2*V617F-induced phosphorylation and downstream JAK-STAT, ERK and AKT pathway activation have not been analyzed. *Aims.* To study the effect of *JAK2*C618R on JAK2 protein phosphorylation and activation *in vitro*, and compare the results with those obtained for *JAK2*V617F or the double mutant *JAK2*V617F+C618R. *Methods.* A full-length wild-type *JAK2* cDNA was amplified by PCR and cloned into a GFP-tagged expression vector. *JAK2*V617F, *JAK2*C618R and *JAK2*V617F+C618R alterations were introduced by direct mutagenesis. Western blotting analysis of JAK2, STAT5, ERK 1 & 2 and AKT protein phosphorylation was performed after transfection of wild-type or mutant constructs into HEK293 cells. The levels of cyclin B1 and cyclin E protein expression were also determined to assess cell cycle progression. *Results.* The phosphorylation of JAK2 and its downstream targets STAT5, ERK 1 & 2 and AKT in the presence of JAK2C618R mutant alone was lower than that of *JAK2*V617F mutant and similar to those of wild-type JAK2. In striking contrast, the double mutant *JAK2*V617F+C618R potentiated the JAK2V617F-induced phosphorylation level of JAK2 (4-fold), STAT5a (3-fold) and AKT (3-fold). A minor increase (1,2-fold) was also observed in the phosphorylation levels of ERK 1 & 2. Furthermore, the *JAK2*V617F+C618R mutant showed a 2-fold and 11-fold increase in cyclin B1 and cyclin E expression, respectively, compared with *JAK2*V617F. *Conclusions.* The presence of *JAK2*C618R alone has no relevant effect in the activation of JAK-STAT, ERK and AKT signalling pathways. However, *JAK2*C618R plays a role as an adjuvant mutation of *JAK2*V617F by promoting a higher activation level of the JAK-STAT and AKT proliferative pathways. Moreover, *JAK2*C618R sinergizes with *JAK2*V617F to increase the levels of cyclin E, suggesting that double mutant cells may proliferate further as a result of cell cycle progression. This work supports the hypothesis that mutations in neighboring residues of 617 are unable to sustain constitutive activation of kinase function of JAK2 unless the *JAK2*V617F mutation is also present. It ultimately provides the rationale for the fact that only *JAK2*V617F is selected in the overwhelmingly majority of PV patients

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0964

DIFFERENTIAL EFFECTS OF TUMOR NECROSIS FACTOR-ALPHA (TNF) IN MYELOPROLIFERATIVE NEOPLASMS (MPN) AND MODULATION BY INHIBITION OF TNF BY JAK2 V617F

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Background. Myeloproliferative neoplasms (MPN) are hematpoietic stem cell diseases encompassing chronic myeloid leukemia (CML), essential thrombocythemia (ET), idiopathic myelofibrosis (IMF), and polycythemia vera (PV). More than 95% of all patients with PV and some 50% of patients with ET and IMF have an activating mutation of JAK2 (JAK2 V617F). Tumor necrosis factor-alpha (TNF) is a pleiotropic cytokine produced by multiple cell types, including macrophages, NK cells, T and B cells. TNF plays a key regulatory role in immune and inflammatory responses. There is also evidence that TNF is involved in the regulation of erythropoiesis. We have previously reported that TNF levels are elevated in mice with retrovirally-induced JAK2 V617F-positve

PV-like MPN and that lack of TNF attenuates MPN in this model. *Methods and results.* We studied the expression of TNF in white blood cells from PV patients (n=43) and normal controls (n=21) using qPCR. PV patients expressed on average 2.5-fold higher levels of TNF than controls (P<0.0004). Upon examination of a panel of human leukemia cell lines (n=8) we found the highest TNF expression in HEL cells, which are homozygous for JAK2 V617F, suggesting that JAK2 V617F may upregulate TNF expression. Consistent with this, we found 6.9-fold higher TNF expression in Ba/F3 cells constitutively expressing EPOR and JAK2 V617F compared to parental cells. In a murine model of retrovirally-induced MPN, TNF expression was found to be two-fold higher in cells expressing JAK2 V617F as compared to JAK2 V617F-negative cells. Exposure of HEL cells or Ba/F3 EPOR JAK2 V617F cells to the JAK2 inhibitor CYT387 resulted in a time dependent 3.3 to 7.5-fold decrease of TNF mRNA as assessed by qPCR. By contrast, TNF mRNA expression was not found to be down-regulated by CYT387 in HL60 cells lacking mutated JAK2. CYT387 was also found to down-regulate TNF mRNA expression in MNC derived from MPN patients. To assess differential effects of TNF on normal *vs.* MPN progenitor cells, we performed clonogenic assays of peripheral blood MNC from normal donors (n=4) and MPN patients (n=9) in the presence of graded concentrations of EPO (0, 0.05, 0.5, 5 Iu/mL) and TNF (1, 10, 100 ng/mL). Intermediate and high TNF (10, 100 ng/mL) caused a dose-dependent reduction of BFU-E and CFU-GM compared to controls. However, in all conditions colony survival in MPN samples was higher compared to normal controls. Low TNF (1 ng/mL) in cultures supported by EPO (0.5 or 5.0 Iu/mL) increased BFU-E formation by MPN cells to 142% of controls, but reduced BFU-E formation by normal MNC to 65%. Analyzing BFU-E and CFU-GM from the clonogenic assays for the presence or absence of the JAK2 V617F mutation, we found, that the proportion of mutated colonies increased in the presence of TNF (10 ng/mL) compared to controls in all MPN patients tested (n=6), suggesting that TNF postively selects for mutated colonies. *Conclusions.* Our data indicate that JAK2 V617F upregulates TNF expression in cell lines and primary MPN cells. Compared to normal progenitor cells, MPN progenitor cells are less sensitive or even stimulated by TNF. These data suggest that JAK2 V617F-induced TNF may contribute to MPN pathogenesis by conferring a growth advantage to MPN over normal cells.

0965

THROMBIN GENERATION IN PLATELET RICH PLASMA IS INCREASED AND CORRELATE TO IMMATURE PLATELETS IN PATIENTS WITH ESSENTIAL THROMBOCYTHEMIA AND POLYCYTHEMIA VERA

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Background. Numerous platelet and leukocyte abnormalities have been identified in Essential Thrombocythemia (ET) and Polycythemia Vera (PV) patients that can contribute to the increased hypercoagulable state typical of these diseases. *Aims.* Our aim is to pursue characterizing the platelet procoagulant properties in both ET and PV patients. *Methods.* In this study we performed the thrombin generation (TG) assay in platelet rich plasma and the PFA-100 assay in whole blood in a group of 45 ET and 44 PV patients to characterize the platelet procoagulant and adhesive properties, respectively. In addition, in the same samples we measured for the first time the peripheral blood immature platelets fraction (IPF) using the Sysmex XE-2100 system. *Results.* Our results show significantly increased levels of IPF, as both absolute count and percentage, in whole blood as well as in PRP samples from PV patients compared to controls, and an increase in IPF, as absolute count, in samples from ET patients. The patients carrying the JAK2V617F mutation had significantly increased IPF compared to controls. PRP from both ET and PV patients generated significantly higher TG compared to control subjects. PV patients on cytoreductive therapy had significantly lower (P<0.05) levels of IPF (9.4×10^9/L) and TG activity (28.5 nM/min) compared to ASA-treated patients (20.2×10^9/L; 33 nM/min respectively). Linear regression analysis showed that a higher IPF count determined higher TG activity (B=0.52; P<0.05) in PV patients. Accordingly, in ET carriers of the JAK2V617F mutation, there was a direct correlation between TG activity and high-IPF% (B=0.2, P=0.07) and/or mean platelet volume (B=0.5, P<0.05). *Conclusion.* This study provides new insight into the characteristics of platelets from PV and ET patients. IPF might be an important contributor to the thrombotic diathesis of these patients, also worth to be tested prospectively for the capacity to identify those patients at high thrombotic risk.

0966

REGULATORY NETWORKS OF JAK2/STAT5 PATHWAY ACTIVITY IN MYELOPROLIFERATIVE NEOPLASIA CELL LINES

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Background. The JAK/STAT signal transduction pathway is frequently altered in hematological cancers and, in chronic myeloproliferative neoplasms (CMN) in particular, the JAK2/STAT5 pathway is most frequently involved. Many studies have focused on the main components of this pathway (JAKs and STATs) and on its negative regulators (PTP, SOCS and PIAS). Although previous studies have identified some genes transcriptionally regulated by the activation of this pathway, other target genes, yet undiscovered, might be playing a role in STAT5-mediated oncogenesis and could represent new therapeutic targets in these neoplasms. *Aims.* Our goal is to identify and characterize new STAT5 target genes, using cell lines where the activation status of the pathway can be modified. This will allow us to improve our understanding of the transcriptional program resulting from JAK2/STAT5 activity, and to uncover new mechanisms that could be modulating this pathway in myeloid cells. Specifically, we will study the existence of hypothetical regulatory networks centered on microRNAs. *Methods.* We used M-07e cell line, where the activity of the JAK2/STAT5 pathway can be induced by IL-3, and the HEL cell line, where the pathway is constitutively activated owing to the presence of the V617F mutation in *JAK2*. We measured by RT-qPCR the expression levels of 64 genes that were predicted as transcriptional targets of STAT5 using bioinformatics tools and expression microarrays. To validate the interaction between STAT5 transcription factor and gene promoters, a chromatin immunoprecipitation assay (ChIP) was performed with a specific anti-STAT5 antibody, and gene promoters were detected in the immunoprecipitated fraction by PCR. Taqman Low Density Arrays (TLDAs) were used to measure the expression of 667 microRNAs after JAK2/STAT5 induction, after treatment with JAK2 and STAT5 inhibitors, and after treatment with a demethylating agent (5-azacytidine). *Results.* We identified ten genes with at least two-fold change in expression levels when the pathway is either induced or inhibited, six of which have confirmed interaction between their promoters and STAT5. We also identified 48 microRNAs that could regulate post-transcriptionally these ten genes and which show significant expression changes in these cell lines. With these data, we have built interaction networks between novel STAT5 target genes and their regulatory microRNAs. *Conclusions.* Our results provide new insights on the transcriptional program triggered by JAK2/STAT5 activation, which is constitutively activated in patients with myeloproliferative neoplasms. The identification of novel transcriptional targets of STAT5 and microRNA-target gene interactions has allowed us to generate new regulatory networks which could lead to a deeper understanding of STAT5-mediated oncogenesis.

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0967

GENE EXPRESSION PROFILE IN JAK2 V617F POSITIVE POLYCYTHEMIA VERA AND ESSENTIAL THROMBOCYTHEMIA RELATED TO RESPONSE TO HYDROXYUREA TREATMENT

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Background. JAK2 V617F has improved the diagnosis and knowledge on the pathogenesis of the classic chronic Philadelphia-negative myeloproliferative neoplasms. Several hypotheses try to explain how a unique mutation can cause three different phenotypes. The current first line of treatment for PV and ET is hydroxyurea, but up to 15 to 25% of patients show resistance or intolerance. *Aims.* We aimed to analyze the differential gene expression profile of JAK2 V617F positive PV and ET, with and without HU treatment. We hoped to identify additional molecular alterations responsible for their divergent phenotypes. In addition, we aimed to correlate these results with clinical and laboratory features, particularly for those patients who had been referred on the basis of their response to treatment, to determine possible mechanisms for a poor response. As a consequence, we hoped to identify alternative

routes for targeted therapy. *Methods.* Twenty-one PV (10 with treatment, 11 at diagnosis), 28 ET (16 with treatment, 12 at diagnosis), eight secondary erythrocytosis patients, and 30 controls were included in the study. cDNA of granulocytes from venous peripheral blood was extracted. A first microarray analysis was performed (Whole Human Genome Microarray Kit, 4 × 44K de Agilent Technologies) and significant genes were validated by low-density quantitative real time PCR array (LDA). Statistical analyses of data were performed using the non-parametric Wilcoxon analysis. Statistical significance was considered when P value was under 0.05. In addition, the bioinformatics tool "Tnasas"was used to construct a prediction modelling. *Results.* Eighty-four genes showed differential expression between ET and PV by microarray analysis. PCR confirmed MMP14 over-expression in PV compared to ET, both at diagnosis and under HU treatment. Thirty-four genes were over-expressed in patients who did not respond to HU. Of these, some participate in proliferative pathways: the MAPK, AKT, Src family kinase (SFK), and JAK2 pathway (see attached Figure). In addition, a minimum error rate 10-gene prediction model was constructed, which included FRMD4B, LRMP, RAF1, FCER1G, ITGAM, CD44, MAPK14, JAK2, EDN1, and SKAP2 genes, over-expressed in the non responder group. JAK2 V617F allele burden was measured at diagnosis and after HU treatment in PV and ET patients. When measured after HU treatment, it diminished in PV, but only significantly in ET (P=0.003). However, the allele burden differences between patients who responded, or those who did not respond to HU, were not statistically significant. *Summary/Conclusions.* To summarize, our results suggest that MMP14 could be involved in the phenotypic divergence between PV and ET. A differential molecular fingerprint of genes distinguishes PV and ET patients who either do or do not respond to HU treatment. JAK2, MAPK14, PIK3CA and SFK genes are over-expressed in patients who do not respond to HU. In our group of patients JAK2 V617F allele burden decreased with HU treatment, however a significant diminution was not observed in response to treatment. On the other hand, patients with no response to treatment over-expressed JAK2. These results should be confirmed with further functional studies.

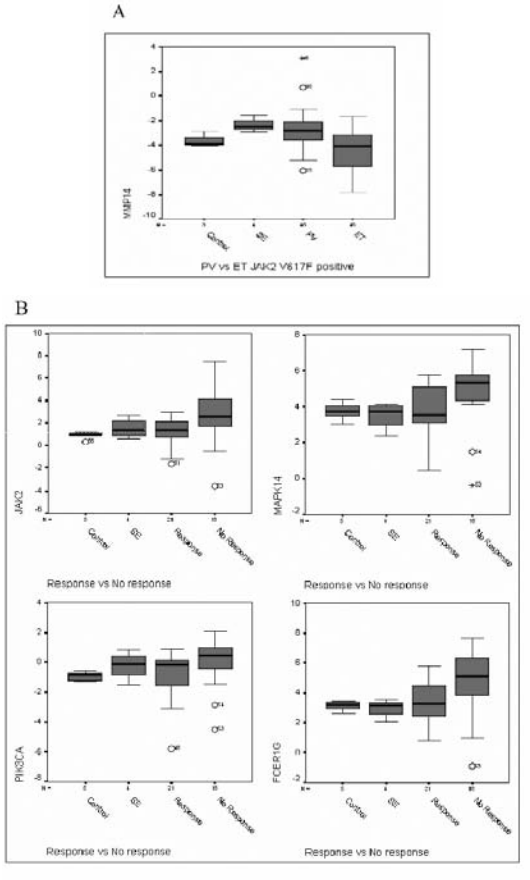

Figure. Gene expression box-plots.

0968

BIOLOGICAL AND FUNCTIONAL CHARACTERIZATION OF *EX VIVO* EXPANDED MESENCHYMAL STROMAL CELLS FROM BONE MARROW OF PATIENTS WITH PRIMARY MYELOFIBROSIS

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Background. Myelofibrosis (MF) is classified among myeloproliferative disorders, being characterized by variable degrees of cytopenia(s), bone marrow fibrosis and extramedullary hematopoiesis. An acquired somatic Janus Kinase 2 (JAK2) mutation (V617F) has been identified in hematopoietic cells of 60% of patients. Mesenchymal stromal cells (MSCs) have been considered for many years a component of marrow stroma with structural support properties only; it is now clear that MSCs substantially contribute to the hematopoietic stem cell niche. *Aims.* The aim of the present study was to characterize the biological and functional properties of MSCs derived from myelofibrotic (MF) patients. *Methods.* MSCs were isolated and expanded *ex vivo* from bone marrow (BM) and from trabecular bone fragments (TBF) of 5 patients , from BM of 2 and from TBF of other 3 patients. MSCs were investigated for clonogenic efficiency (CFU-F), proliferative capacity (population doubling-PD), morphology, immunophenotype (flow cytometry), differentiation potential (histological staining and spectrophotometry), genetic characterization (comparative Genomic Hybriditation, array CGH) and molecular analyses (Polimerase Chain Reaction, PCR). The ability of patients' MSCs to suppress *in vitro* proliferation to mitogens (PHA, OKT3) of both autologous and allogeneic lymphocytes was also assessed at different MSC:PBMC ratios. Results were compared to those obtained in BM-MSCs from 9 healthy donors (HD). *Results.* CFU-F frequency was comparable in MF- (median±SD: 5.37±1.1 for BM and 6.84±1.9 for TBF, respectively) and HD-MSCs (median±SD: 5.29±0.64). Proliferative capacity of BM-MSCs (mean cumulative PD from P1 to P4: 8.42) and TBF-MSCs (mean cumulative PD from P1 to P4: 8.48) of patients was comparable to that of HD-MSCs (mean cumulative PD from P1 to P4: 8.24). MF-MSCs displayed the typical spindle-shaped morphology and were able to differentiate into both adipocytes and osteoblasts. In particular, the osteogenic differentiation capacity of TBF-MSCs resulted higher than that observed in BM-MSC from the same 5 patients as demonstrated by spectrophotometry (mean±SD: 882±873; 264±354 µg/mL, respectively). Surface immunological markers did not differ between MF- and HD-MSCs. MF-MSCs ceased their growth at variable passages (BM-MSCs from P8 to P14 and TBF-MSCs from P10 to P14) and entered senescence, without any change in morphology/proliferation rate. Results of the array-CGH analysis showed that MF-MSC expanded *in vitro* did not exhibit chromosomal abnormalities. MSCs were tested negative for JAK2V617F mutation even in those patients with hematopoietic cells positive. Both in the autologous (MF-MSCs/MF-PBMCs) and allogeneic (HD-MSCs/MF-PBMCs) setting, proliferation of PBMCs was reduced up to 81% and 93.4%, respectively, and resulted to be comparable to that obtained in the setting HD-MSCs/HD-PBMCs (maximum inhibition 95%). *Conclusions.* Further studies are needed to investigate if the biological and functional properties of MF-MSCs are involved in the etiopathological mechanisms responsible for this myeloproliferative disorder.

0969

ASSOCIATION OF POLYMORPHISMS IN THE JAK2 LOCUS WITH A PREDISPOSITION TO POLYCYTHEMIA VERA DEVELOPMENT, AND THE ACQUISITION OF THE JAK2V617F MUTATION IN CHRONIC MYELOPROLIFERATIVE NEOPLASMS

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Background. Myeloproliferative neoplasms (MPN) are heterogeneous blood diseases characterized by clonal hematopoiesis, chronic excessive production of differentiated blood cells and increased risk for thrombosis and secondary leukemic transformation. A recurrent unique somat-

ic clonal mutation in the JAK2 kinase gene, JAK2V617F, has been identified in the majority of individuals with MPN (65-97% Polycythemia Vera (PV), 23-95% primary myelofibrosis (PMF), 41-57% essential thrombocythemia (ET)). This mutation is, however, not found in some patients indicating that is a secondary event. Three recent studies identified several polymorphisms associated with the acquisition of the JAK2V617F mutation. *Aims.* Analyze the association of three polymorphisms, rs10974944, rs12340895 (JAK2) and rs12500918 (4q31), with the risk to develop MPN, to acquire the JAK2V617F mutation and the MPN phenotype. *Methods.* Our study was performed on 525 Caucasian subjects: 356 controls and 169 patients with MPN: 46 with PV, 107 with ET and 16 with PMF. 56 patients were JAK2 V617F negatives and 113 positives. Genotyping of these polymorphisms were determined by validated TaqMan SNP Genotyping Assay and analyzed with the SNPstats software. *Results.* There was a significant association between the two JAK2 SNPs rs10974944 and rs12340895 (OR=2.87, 95% CI=2.16-3.82, P-value=<0.0001; OR: 2.78, 95% CI=2.09-3.69, P-value=<0.0001) with the acquisition of the JAK2 V216F mutation. Also, we found a significant association between the haplotype formed by the G allele of rs10974944, the G allele of rs12340895 and the A allele of rs12500918, with the susceptibility to acquire JAK2V216F mutation (OR=3.63, 95% CI=2.23-5.92, P-value=<0.0001). Moreover, we observed a significant association between development of MPN and rs10974944 (OR=2.10, 95% CI=1.59-2.78, P-value=<0.0001) and rs12340895 (OR: 2.06, 95% CI=1.56-2.72, P-value=<0.0001). In addition, the presence of genotype G/G of rs10974944 and G/G of rs12340895 was associated with the development of PV (OR=8.54, 95% CI=4.35-16.76, P-value=<0.0001 and OR=8.42, 95% CI=4.24-16.74, P-value=<0.0001, respectively). However, we don't find the reported association of the rs12500918 SNP on chromosome 4 and the MPN phenotype. *Conclusions.* Ours results confirmed that presence of the G allele of rs10974944 and rs12340895 are predisposing factors to acquire the JAK2V216F mutation. In addition, we demonstrated a significant association of genotype G/G of rs10974944 and G/G of rs12340895 and the development of PV. Our results support previous data suggesting that germline variations are important contributor to MPN phenotype and predisposition.

0970

BONE MARROW STROMA-MEDIATED PARACRINE INHIBITION OF JAK2 INHIBITOR-INDUCED APOPTOSIS OF JAK2V617F-MUTATED CELLS

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Signals emanating from the bone marrow niche are suspected to support the growth of malignant clones in patients with myeloproliferative neoplasms (MPN), but also to protect them from targeted therapies. Little is known about the crosstalk between MPN clones and the marrow stroma. We have streamlined a co-culture platform designed to interrogate the interplay between JAK2V617F-positive cells and the stroma microenvironment in the context of JAK2 inhibitor therapy. Treatment with atiprimod, a potent JAK2 inhibitor, causes marked apoptosis of both human (SET2) and mouse (FDCP-EpoR) JAK2V617F-positive cells but this effect was abrogated when the latter were directly co-cultured (cell-on-cell) for 48h with human marrow stromal cell lines (HS5, NK.tert, or TM-R1). Co-culture with stromal cells hampered the ability of atiprimod to inhibit the phosphorylation of JAK2, STAT3, and STAT5. Notably, the protective effect of stromal cells was also observed in non-contact co-culture assays (cell lines separated by 0.4 μm micropore membranes), suggesting a paracrine effect. Cytokine profiling of supernatants generated in non-contact co-cultures in the presence or absence of atiprimod identified IL-6, FGF, and CXCL10/IP10 as elevated during atiprimod treatment. Exposing stromal monolayers to neutralizing antibodies against IL-6, FGF, or CXCL10/IP10 prior to coculture with JAK2V617F-positive cells ablated the protective effect of stromal cells and markedly increased atiprimod-induced apoptosis. Our results suggest that specific humoral factors secreted by stromal cells protect MPN clones from JAK2 inhibitor therapy, thus underscoring the importance of targeting the marrow niche in MPN for therapeutic purposes.

0971

C-CBL, CBL-B AND CBL-C ANALYSIS IN BCR-ABL1 NEGATIVE CHRONIC MYELOPROLIFERATIVE NEOPLASMS

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Background. BCR-ABL1 negative chronic myeloproliferative neoplasms (CMPNs) are a heterogeneous group of clonal hematological malignancies. Some genetic aberrations have been described to cause these diseases, most of them activating tyrosine kinase (TK) genes. However, in last years, different groups have described mutations in other molecules involved in the same signaling pathways that could have important roles in the disease. One of them is the CBL (Casitas B-lineage Lymphoma) family of E3-Ubiquitin-ligase proteins encoded by three genes: *C-CBL*, *CBL-B* and *CBL-C*. CBLs are involved in the negative regulation of several tyrosine-kinases, as *EGFR*, *FGFR* or *SYK*. Several groups have demonstrated that mutations in the RING Finger domain of *C-CBL* result in deregulation of downstream targets of this protein. *Methods.* We have used dHPLC to detect sequence mutations on samples from 382 *BCR-ABL1* negative CMPN patients, 145 *V617FJAK2* negatives and 237 positives. We included 20 samples from healthy individuals as controls. *Results.* We have found four non-described mutations in *C-CBL*, three of them located in the *RING Finger domain* (T402H, C416W, P417R), and two in the *Proline-Rich domain* (S675C and A678V). T402H, P417R and S675C were found in *V617FJAK2* positive patients. We have found and additional change (R462W) in the RING Finger domain of *CBL-B*, detected in a patient with *V617FJAK2* positive polycythemia vera. *Conclusion.* Our results suggest that mutations in *CBL* and *JAK2* genes are not exclusive events.

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0972

SUPPRESSOR OF CYTOKINE SIGNALING 3 IS DOWN-REGULATED BY BCR-ABL AND RESULTS IN LOSS OF JAK2 V617F IN A PATIENT WITH CONCURRENT BCR-ABL TRANSLOCATION AND JAK2 V617F MUTATION

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Background. The JAK2 V617F mutation, found in approximately 95% of polycythemia vera (PV) cases results in constitutive phosphorylation of JAK2 kinase while the BCR-ABL fusion gene is observed in chronic myeloid leukemia (CML) and results in dysregulated ABL kinase activity. Both these mutations rarely occur together and only a few cases of Ph^+, JAK2 $V617F^+$ myeloproliferative neoplasm (MPN) have been reported. We have recently reported such a case and observed a reciprocal relationship between JAK2 V617F and BCR-ABL, whereby an increase in BCR-ABL mRNA levels resulted in disappearance of JAK2 positive cells and reappearance of the JAK2 $V617F^+$ clone once BCR-ABL had been suppressed by imatinib mesylate (IM). The co-existence and interaction between JAK2 V617F, genes involved in the JAK/STAT signaling pathway, and BCR-ABL is yet to be studied. We sought to examine this relationship from serial archived samples collected over a 4-year period from this unusual patient. *Methods.* RNA was extracted from archived cell lysates obtained at different time-points from the patient. The patient had an initial clinical presentation consistent with PV, during which he was JAK2 $V617F^+$ with low level BCR-ABL transcripts (period-1). He subsequently progressed to a picture consistent with CML, and at peak levels of BCR-ABL transcripts, JAK2 V617F mutations was not detectable (period-2). With commencement of IM, JAK2 V617F was detectable again with gradual decline of BCR-ABL transcripts (period-3). The patient achieved major molecular remission in 24 months from commencement of IM. We investigated the expression of 84 selected genes involved in the JAK/STAT pathway in 4 representative samples, one collected during period-1, two at period-2 and one at period-3. Gene expression analysis was performed using SYBR-Green quantitative RT-PCR on 384-well microplates, measured in triplicates on a real-time PCR analyser. Significant differentially expressed genes between periods-1 and 3 combined and period-2 were identified using Significant Analysis of Microarray (SAM). Identified genes were subsequently validated by quantitative RT-PCR using specific primers on a total of 7 samples obtained at varying periods. *Results.* Twelve genes showed significant down-regulation during the period when BCR-ABL

transcripts was high with absence of JAK2 V617F (period-2). Of specific interest was the suppressor of cytokine signaling 3 (SOCS3), which showed 110-fold down-regulation during loss of JAK2 V617F and high expression of BCR-ABL. Further gene expression analysis using SOCS3 specific primers on seven samples from the same patient, confirmed the marked decline of SOCS3 gene expression in samples with increased BCR-ABL mRNA burden and absence of JAK2V617F. SOCS3 expression was also shown to be significantly higher in JAK2 V617F$^+$ samples with low BCR-ABL mRNA level as compared to normal subjects and Ph$^+$ CML. *Conclusion.* Our study reiterates that JAK2 V617F escapes negative regulation by SOCS3. Indeed, SOCS3 may play a permissive role in maintaining the JAK2 V617F clone, as abolishing SOCS3 expression in the presence of BCR-ABL is associated with loss of JAK2 V617F allelic burden. Furthermore, BCR-ABL transcripts are capable of down-regulating SOCS3 expression which may partly contribute to its anti-apoptotic potential.

0973

THERAPY RELATED GENE EXPRESSION OF HEMATOPOIETIC PROGENITOR CELLS IN CHRONIC MYELOPROLIFERATIVE NEOPLASMS

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The gene expression profiles in chronic myeloproliferative neoplasms (CMN) may reveal gene markers of potential clinical utility for the diagnosis and prediction of response to therapy. Investigating the extent to which these genes participate in the complex molecular and cellular mechanisms of CMN, by microarray analysis, will likely lead to new insights. The JAK2V617F mutant allele burden can determine the laboratory and clinical characteristics of CMN associated to various genes and signaling pathways, described by microarray analysis. The quantity of CD34$^+$ hematopoietic progenitor cells, determined by flow cytometry, and leukocytes have been elevated in JAK2V617F mutation homozygous *vs.* heterozygous patients with polycythemia vera (PV), as well as in JAK2V617F homozygous and heterozygous *vs.* no mutation patients with primary myelofibrosis (PMF). In addition, the thrombocytes levels were reduced in JAK2V617F heterozygous (1003×10^9/L) *vs.* no mutation (1369×10^9/L) patients with essential thrombocythemia (ET). Splenomegaly was more prominent in homozygous patients with PV and PMF, whereas the presence of JAK2V617F mutation delayed the age beginning of PMF. The 118 genes had statistically significant difference in expression ($P<0.05$) between controls and ET, as measured by microarray analysis in CD34$^+$ hematopoietic progenitor cells. The presence of heterozygous JAK2V617F mutation increased 4 fold the quantitative difference in genes expression. The prominent genes with increased expression were OAZ1, MTPN, SOD2, HLA-G, WDR1, whereas with reduced expression were ADAMTSL3, HIF3A, GNGT1, SUCLG2, NFS1 in ET heterozygosity. The 175 genes had statistically significant difference in expression between controls and heterozygous PV, while the homozygous form of JAK2V617F mutation doubled the difference in genes expression. The outstanding genes with increased expression were NMI, SOD2, TALDO1, GNAI2, KIF2A whereas with reduced expression were MLLT4, HIF3A, NFS1, ADAMTSL3 and DGKB in patients with PV. The genes with increased expression in PMF *vs.* controls were NMI, TNFRSF1A, NCOA4, PDCD10, KIF2A whereas with reduced expression were CDRT1, RUNDC2B, MCC, S100PBP and SDHAP3 in CD34$^+$ hematopoietic progenitor cells. Hydroxyurea and Interferon-alpha are drugs used in therapy of CMN patients. Hydroxyurea treatment stimulated expression of G-protein coupled receptors related genes activated by cAMP/PKA pathway (ADIPOQ, 3.1 fold), PI-3 kinase pathway (AKT1, 1.6 fold), NO/cGMP pathway (PRPF18, 2.6 fold), PKC pathway: JUNB and IL-8 (1.5 and 3.7 fold, respectively), MAP kinase pathway: SERPINE1, JUN and MAX (1.2, 1.6 and 4.3 fold, respectively) and JAK-STAT pathway: SOCS1, HSD3B1 (2.7 and 1.8 fold, respectively). The gene expression of PRPF18 was increased in PV, MAX in PV and PMF, while IL-8 gene expression was increased in ET patients. Regarding IFN alpha signaling pathway related genes, JAK1 has been elevated in PV patients with JAK2V617F mutation; TYK2, STAT1, STAT2, IFNA2 have been elevated in all CMN. The increased expression of OAZ1, SOD2, NMI, TALDO1 genes and decreased expression of ADAMTSL3, MLLT4, HIF3A genes, observed in all CMN, have a potential for a cellular transformation and may contribute to myeloid leukemia development. The presented drug related genes, especially IL-8 and SOCS family members, contribute principally in cancer cells response and adaptability to chemotherapy.

0974

MUTATIONS OF TET2, ASXL1, CBL AND IDH1 IN MYELOFIBROTIC TRANSFORMATION OF ESSENTIAL THROMBOCYTHEMIA AND POLYCYTHEMIA VERA

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Background. Acquired mutations in the TET2 and ASXL1 gene have been described in approximately 14% and 8% of myeloproliferative neoplasms (MPN), respectively. In addition, mutations in the CBL and IDH1 gene, as well as TET2 and ASXL1, have been reported as genetic events associated to secondary acute or chronic myeloid transformation in MPN. A variable percentage of ET and PV patients develop myelofibrotic transformation during the evolution of the disease, although the genetic events that underlie myeloid transformation are still unclear. *Aim.* To analyze the presence of TET2, ASXL1, CBL and IDH1 mutations in a cohort of ET and PV patients who underwent transformation to myelofibrosis (MF). *Patients and methods.* Seventeen ET and PV patients with myelofibrotic transformation were assessed for mutations in: TET2 (whole exome), ASXL1 (exon 12), CBL (exons 8&9) and IDH1(R132). The mutational analysis of the aforementioned molecular markers was performed by direct sequencing using cDNA from purified granulocytes. All the samples analyzed were obtained at the myelofibrotic phase of the disease. *Results.* From the 17 patients analyzed, 3 of them (2 post-PV MF, 1 post-ET MF) presented mutations in the TET2 gene. The three TET2 mutations were: P463Lfsx23, T229NfsX25 and Q706X. Moreover, 2 additional patients (1 post-PV MF, 1 post-ET MF) presented mutations in the ASXL1 exon 12. The ASXL1 mutations were: Y591X and L765AfsX11. The 2 post-ET MF patients were JAK2V617F-negative whereas the 3 post-PV MF patients harbored the JAK2V617F mutation in homozygosis. CBL and IDH1 mutations were not detected in any of the patients analyzed suggesting that these genes might not be involved in the myelofibrotic evolution of a MPN. Overall, pathogenic mutations were observed in 29.4% of MF patients. In the patient with a TET2 mutation, the Q706X mutation was detected in an advanced phase of the myelofibrosis but not in a sample corresponding to the initial phase of the transformation. *Conclusions.* 1-TET2 and ASXL1 pathogenic mutations are present in nearly 30% of ET and PV patients who develop myelofibrotic transformation. 2-CBL and IDH1 mutations might not be genetic events involved in the myelofibrotic transformation of ET and PV patients. 3-TET2 mutations can be acquired during the evolution of a MPN.

0975

SCREENING FOR PDGFRA FUSIONS IN EOSINOPHILIA IS HINDERED BY THE EXISTENCE OF MULTIPLE PDGFRA TRANSCRIPTS

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Background. Hypereosinophilic syndromes comprise a heterogeneous group of hematologic malignancies with persistent eosinophilia and eosinophil infiltration as main features. The lack of genetic markers in a large number of cases makes it difficult to distinguish these primary or clonal diseases from reactive or secondary eosinophilias. However the discovery of genetic alterations in *PDGFRA*, *PDGFRB* and *FGFR1* associated with the development of some eosinophilia cases has allowed to improve the classification and treatment of these diseases. *FIP1L1-PDGFRA* fusion is the most common recurrent genomic aberration found in myeloproliferative neoplasms with eosinophilia, although five more *PDGFRA* fusion genes have been described to date. Additionally, *PDGFRA* abnormalities have also been found on gastrointestinal tumours, glioma/glioblastoma, prostate cancer metastasis, testicular germ cell tumors, hepatocellular carcinoma and systemic mastocytosis. Previous reports looking for *PDGFRA* fusions have found *PDGFRA* TK domain overexpression at mRNA level not explained by the existence of putative *PDGFRA* transcripts. However we hypothesized that this differential expression of *PDGFRA* regions could be due to the expression of alternative and shorter *PDGFRA* transcripts from an alternative promoter, described years ago in teratocarcinoma. *Aims.*

Confirm the existence of *PDGFRA* alternative transcripts in hematological tissue. Find a relation between *PDGFRA* reported TK coding region overexpression and alternative transcripts expression. In case this proves real, develop a methodology to properly discriminate putative *PDGFRA* fusions and expression from different promoters. *Methods.* We studied overexpression of *PDGFRA*'s TK domain coding region in HES samples by competitive multiplex RT-PCR as described previously. The existence of several transcription start sites (TSSs) was assessed using 5' RACE on commercial bone marrow total RNA. Identification of different *PDGFRA* transcripts in peripheral blood samples was carried out using specific RT-PCR designs for each transcript. Real time quantitative PCR (RQ-PCR) assays were designed to evaluate expression of three different regions of *PDGFRA* present only in certain transcripts or fusion genes. *Results.* We confirmed the existence of an internal TSS in *PDGFRA*'s intron 12. We also found that expression from this TSS is common in peripheral blood of healthy individuals. Expression from *PDGFRA*'s internal TSS was found in the samples were TK coding region overexpression had been found. We found that our three-assay RQ-PCR methodology was able to distinguish expression from exon 1 and intron 12 TSSs and the presence of putative *PDGFRA* fusion genes. *Conclusions.* Our work has revealed that alternative *PDGFRA* transcripts should be taken into account when studying *PDGFRA* expression aberrations, such as fusions. The existence of these transcripts has allowed us to explain the TK coding region overexpression observed in HES cases where PDGFRA fusions were not found. Our RQ-PCR approach proved adequate for detecting and discriminating expression from both TSSs and *PDGFRA* fusions.

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0976

DNA METHYLATION IN POLYCYTHEMIA VERA AND ESSENTIAL THROMBOCYTHEMIA

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Background. DNA methylation plays a critical role in regulation of gene expression in development, differentiation and diseases. Aberrant DNA methylation has been demonstrated to participate in pathogenesis of haemathologic malignancies, as MDS and NHL. An aberrant methylation pattern has been described in SOCS as a potential contribution to pathogenesis and differential phenotype in MPN. *Aims.* The purpose of this study was to study DNA methylation in classic chronic Ph negative Myeloproliferative Neoplasms (MPN), Polycythemia Vera (PV) and Essential Thrombocythemia (ET) in order to identify possible specific methylation signatures related to pathogenesis and susceptible of potential therapeutic targets. *Methods.* 48 samples, 36 from patients diagnosed of classic chronic Ph negative MPN (12 ET JAK2 V617F positive, 12 ET JAK2 V617F negative, 12 PV JAK2 V617F positive) and 12 from healthy donors were included in the study. Diagnosis was established according to WHO criteria. Peripheral venous blood samples were collected in EDTA and immediately processed extracting DNA. Bisulphite conversion of the DNA was performed using the "Zymo EZ DNA Methylation Kit"according to the manufacturer's procedure (Zymo Research, Orange, CA). The processed DNA samples were hybridized to a beta-test version of the "HumanMethylation27 DNA Analysis BeadChip"(Illumina Inc.). This array was developed to assay 27,578 CpG sites selected from more than 14,000 genes. A bioinformatics tool GenomeStudio (Illumina Inc.) was used to statistical analysis applying the following criteria: Delta Beta > 0.3 and Mann-Whitney statistic corrected by false discovery rate (FDR), Differential Score > 20.0. A dichotomic analysis among the four groups was performed. *Results.* After a dichotomic pair matched analyses among the four referred groups was performed, only comparison between JAK2 V617F positive PV and ET, and between JAK2 V617F positive PV and controls generated a different methylation pattern. On the other hand all groups showed a very homogeneous methylation pattern (see attached Figure). VCY gene showed a differential methylation pattern, with clear statistical significance, between JAK2 V617F positive PV and ET. ZNF577 gene showed a differential methylation pattern between JAK2 V617F positive PV and controls. *Conclusions.* VCY gene, located in Y chromosome, is involved in sexual differentiation. For this reason it seems difficult to establish a link with pathogenesis in MPN. ZNF577 gene, methylated in JAK2 V617F positive PV in comparison to controls, encodes for a zinc finger family member protein, with to date undetermined physiologic function. According to our results aberrant methylation pattern does not seem to play a crucial role in MPN pathogenesis.

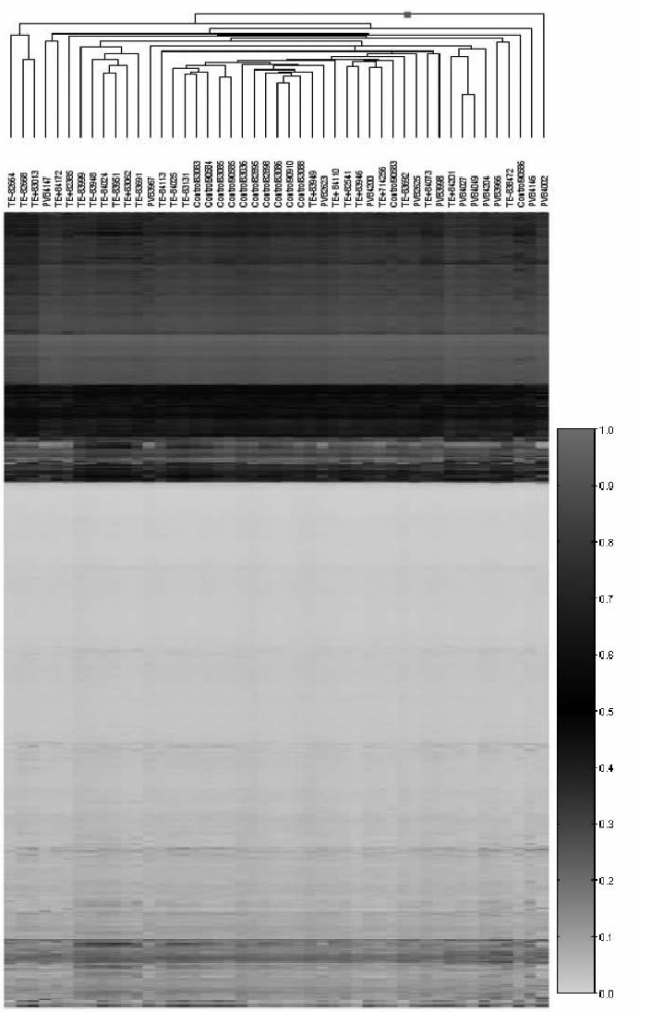

Figure. Heat Map of methylation profile in MPN.

Myeloproliferative disorders - Clinical

0977

JAK2V617F MUTATIONAL STATUS BUT NOT ALLELE BURDEN PREDICTS SURVIVAL AFTER ALLOGENEIC STEM CELL TRANSPLANTATION FOR MYELOFIBROSIS

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Background. Allogeneic stem cell transplantation (ASCT) after reduced-intensity conditioning (RIC) has become a reasonable treatment option for patients with advanced myelofibrosis. The role of characteristic molecular genetic abnormalities such as JAK2V617F on outcome of ASCT is not yet elucidated. *Aims and methods.* In 139 out of 162 myelofibrosis patients with known JAK2V617F mutation status who received ASCT after RIC the impact of JAK2 genotype, JAK2V617F allele burden and clearance of mutation after ASCT was evaluated. *Results.* Overall survival was significantly reduced in multivariate analysis in patients harbouring JAK2 wild-type (HR 2.23, P=0.007) in comparison to JAK2 mutated patients, due to a non-significant higher treatment-related mortality (31% *vs.* 19%, P=0.1) and relapse incidence (30% *vs.* 21%, P=0.2). No significant influence on survival was noted for the mutated allele burden analyzed either as continous variable or after division in quartiles. Achievement of JAK2V617F negativity after ASCT was significantly associated with a decreased incidence of relapse (HR 0.22, P=0.04). In a Landmark analysis, patients who reached an undetectable JAK2 mutation level in peripheral blood 6 months after ASCT had a significant lower risk of relapse (5% *vs.* 35%, P=0.03) than patients who remained positive. *Summary and Conclusion.* We conclude that JAK2V617F mutated status but not allele frequency was associated in an improved survival, and that its rapid clearance after allografting reduced the risk of relapse.

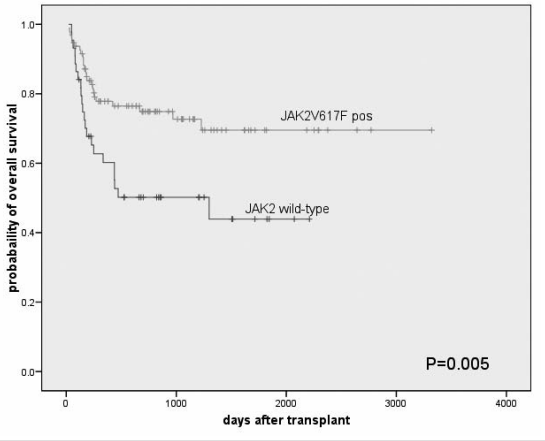

Figure. Propability of overall survival acoording to JAK2.

0978

THE MYELOPROLIFERATIVE NEOPLASM SYMPTOM ASSESSMENT FORM (MPN-SAF): PROSPECTIVE VALIDATION OF AN EVIDENCE BASED MPN SPECIFIC INSTRUMENT

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Background. Symptomatic burden in myeloproliferative neoplasms (MPNs) is present in over 70% of MPN patients (Mesa *et al.* Cancer 2007), and may be improved by JAK2 inhibitors. Given no current instrument assesses the range of MPN symptoms we previously created and validated the Myelofibrosis Symptom Assessment Form (MF-SAF) (Mesa *et al.* Leukemia Research 2009). We sought to validate an expanded instrument (MPN-SAF) to assess symptoms in myelofibrosis (MF), essential thrombocythemia (ET) and polycythemia vera (PV). *Methods.* MPN-SAF: Using the previously validated MF-SAF as a base instrument, we added several key additional symptoms previously identified as present in all subtypes of MPNs including headaches, concentration, dizziness, extremity tingling, insomnia, sexual problems and mood changes on a 0 (absent) to 10 (worst-imaginable) scale. Validation. The MPN-SAF was administered jointly with the EORTC-QLQ-C30 as the co-validation instrument as well as a physician assessment of patient symptoms, and the patient's perception on the clarity, ease of completion, and comprehensiveness of the MPN-SAF. *Results.* Patients: 76 patients were prospectively enrolled (ET (N=14;18%), PV (N=17; 23%) and MF (N=45; 59%)) a median of 4 years (range:0-43) from their diagnosis. Patients were of a median age (65; range 26-88 years) and gender (50% females) typical of the disease. 79% (N=60) had received some form of cytoreductive therapy for their disease. MPN-SAF *Results.* Consistent with our prior symptomatic inventory trials we found the majority of MPN patients to be symptomatic with the majority of patients (>50%) in all prior areas of questioning except the advanced MF symptoms of fever (13%), weight loss (36%), and bone pain (42%). New items for the MPN-SAF were common including headache (45%), decreased concentration (59%), dizziness (54%), numbness (50%), insomnia (71%), sad mood (60%), and challenges with sexuality (57%). Symptoms were present across the subtypes of MPNs with MF being more noteworthy for abdominal symptoms, inactivity, fever, and weight loss (P<0.05 for all). Comparison to EORTC-QLQ-C30: Pearson correlations between MPN-SAF individual symptom scores and the EORTC-QLQ C30 showed excellent correlations with co-validation questions including fatigue, pain, headache, insomnia, early satiety, and sad mood (all P<0.001). Correlations with EORTC-QLQ-C30 subscales demonstrated excellent correlations between MPN-SAF measurements of fatigue (and EORTC physical function subscale), early satiety, abdominal symptoms, inactivity, decreased concentration, insomnia, mood (and EORTC emotional functioning subscale), and challenges with sexuality (Multiple correlations) (all P<0.001). Additionally the MPN-SAF single item assessment of overall quality of life was highly correlated (P<0.001) with all EORTC-QLQ-C30 subscales.Correlation of MPN-SAF to Physician Perceptions: The MPN-SAF results correlated well with physicians perception of fatigue, pruritus, night sweats, fever, weight loss, and bone pain (all P<0.001). Patient Assessment of MPN-SAF: The majority found the MPN-SAF easy to understand (98%) and "addressed most of my MPN symptoms"(96%). *Conclusions.* The MPN-SAF is comprehensive, clear, simple to administer, and valid instrument using of 19 separate MPN associated symptoms. Serial validation on current clinical trials, as well as validated translations into multiple languages is ongoing to establish the MPN-SAF as a uniform symptom assessment tool for MPN patients on clinical trials globally.

0979

HYDROXYUREA IN ESSENTIAL THROMBOCYTEMIA: RATE AND CLINICAL RELEVANCE OF RESPONSES BY EUROPEAN LEUKEMIANET CRITERIA

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A standardized definition of response by cytoreductive therapy in Polycythemia Vera (PV) and Essential Thrombocythemia (ET) was recently provided by the European LeukemiaNet (ELN) investigators. These experts reached a consensus in defining three categories of response. In ET, clinico-hematological (CH) response included: 1) Complete response (CR), defined as platelet count less than or equal to 400×10^9/L, no disease-related symptoms, normal spleen size, and white blood cell count less than or equal to 10×10^9/L; 2) Partial response (PR), recognized in patients who did not fulfil the criteria for CR, and with a platelet count less than or equal to 600×10^9/L or a decrease greater than 50% from baseline; 3) non response (NR), classified as any response that did not satisfy PR criteria. However, it should be mentioned that these definitions resulted from a consensus process and their clinical significance is unknown. To provide estimates and clinical correlation of responses according to these criteria we retrospectively examined 416 ET patients (median age 66 years, 154 male/262 female) treated with Hydroxyurea (HU) for at least 12 months with the goal to keep platelet number less than 600×10^9/L. The first purpose was to estimate the frequency of CH responses, as defined by the ELN. CR rate progressively

increased over time and reached the maximum after 12 months (25%). In contrast, the majority of our treated patients (58%) achieved PR. In this subgroup, reasons for PR classification were due to persistence of leukocytosis (n=28/242, 11%) or incomplete recovery of microvascular symptoms or splenomegaly (n=37/242, 15%). No responders were 72/416 (17%). The probability of being a responder was higher in patients with age more or equal to 60 years and JAKV617F status, confirming a major drug sensitivity in this latter group. The second purpose of our analysis was to correlate the ELN responses with clinical outcomes. After a median follow-up of 3.9 years, we registered 23 deaths, 16 haematological transformations in post-ET myelofibrosis (MF)/acute myeloid leukaemia (AML) and 27 major thrombotic events (rate 1.66% patient/year). The rate of post-ET MF and AML was comparable to the HU arm of PT1 trial (0.68 *vs.* 0.84% patient/year, respectively). These events were not associated with the achievement of haematological responses. In univariate and multivariate analysis, age and previous thrombosis were independently associated with major vascular events, while the achievement of ELN responses, at 12 months of therapy, did not predict future vascular complications. Different levels of platelet count (<400 or <600×10^9/L) at 12 months of therapy did not influence the occurrence of future thrombosis. In contrast, patients with more than 10×10^9/L leucocytes had a significant higher rate of vascular complications independently from the other meaningful variables (P=0.015). In conclusion, our findings would indicate that the response of platelet count does not seem of prime relevance in the definition of ELN response, since the correlation with vascular events is more affected by leukocyte than platelet count. This notion should be considered in future clinical trials with novel JAK2 inhibitors.

0980

SINGLE AGENT BEVACIZUMAB FOR MYELOFIBROSIS: RESULTS OF AN INTERNATIONAL MYELOPROLIFERATIVE DISORDERS-RESEARCH CONSORTIUM (MPD-RC) TRIAL

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Background. Myelofibrosis (MF) has no approved or effective medical therapy, and has been associated with the development of very significant intra-medullary angiogenesis potentially from an increased release of growth factors such as vascular endothelial growth factor (VEGF) and other cytokines. We conducted a 2-stage multicenter phase II trial of the VEGF inhibitor bevacizumab through the clinical trial branch of the MPD-RC in patients with MF. *Methods.* Patients: Symptomatic, relapsed/ refractory and intermediate/ high risk MF patients (both primary and post ET/PV MF) were eligible. Although no minimal requirements for hematologic parameters at study entry, patients with either recent vascular events or surgery were not eligible. Therapy: Patients received bevacizumab at a dose of 15 mg/kg intravenously on day 1 of a 21-day cycle for 4 cycles. Patients were evaluated for response after cycle 4 and 8. Non-responders would go off study after cycle 8, responders could receive up to 17 cycles. *Results.* Enrollment: 13 patients (10 males: 77%) were enrolled beginning May 2008 (8 PMF, 3 Post ET MF, 2 Post PV MF) of an age typical for the disease (median 71 years: range 48-84). Anemia was universally present median hemoglobin of 9.2 g/dL (range 3.0-10.7, 5 erythrocyte transfusion dependent) and six patients (46%) had an MF associated molecular mutation (5 JAK2-V617F, 1 MPL-515). Safety: Eight patients (61%) experienced a moderate to severe adverse event that was attributable to the bevacizumab therapy. Serious events (CTC 3.0 Grade 3 or 4) at least possibly related to therapy included change in LFTs (n=2), myelosuppression (n=2), pain (n=2), infection (n=2), diarrhea (n=1), heart failure (n=1), and other (n=3). One event possibly related to treatment led to a fatality in a patient with multiple co-morbidities and history of cardiac disease. No hemorrhagic or thrombotic events were observed. Response: 11 patients were eligible for response assessment as 2 patients withdrew consent prior to initiating therapy. A total of 40 cycles of bevacizumab have been given of which 5/11 (46%) patients received 4 or more cycles and were evaluable for response. Three patients continued on the second phase of therapy (cycles 5-8) for "stable disease"; one of these 3 patients completed the 8th cycle and stopped secondary to lack of response. No patient has had an objective response. Reasons for termination of therapy included toxicity (n=5), disease progression (n=1), removal from study (n=4), other (n=1). Analysis of the first stage, of this 2 stage trial design, demonstrated inadequate responses occurred for trial continuation to the second stage. Additionally, review by the external MPD Data Safety Monitoring Committee supported premature termination of the trial based on observed toxicities. Correlative biomarker studies are being conducted. *Conclusions.* Bevacizumab at the standard dose and schedule was poorly tolerated in patients with MF. Effects of treatment on biomarker profiles may be informative to guide further studies. Bevacizumab at lower doses, or other angiogenesis inhibitors, alone or in combination, may still be worthy of future study.

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0981

LONG TERM PATIENT-ADJUSTED MAINTENANCE SCHEDULE OF MEPOLIZUMAB IS SAFE AND EFFECTIVE IN HYPEREOSINOPHILIC SYNDROME (HES)

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Background. Steroids are currently considered as the gold standard treatment of FIP1L1/PDGFRalpha negative HES. However, such approach has limited efficacy and possible side effects if chronically used. Mepolizumab is an anti-IL-5 monoclonal antibody recently shown to be effective in reducing peripheral blood eosinophilia and controlling symptoms. However, long term results have not been assessed yet. *Aim.* In this study we aimed to assess the long term effects of Mepolizumab in treatment-requiring HES patients. *Methods.* We treated 7 patients with HES refractory or intolerant to steroid. Mepolizumab was administered at the dose of 750 mg ev monthly until response. Maintenance therapy was subsequently given according to clinical indications at the same dose. Four patients had a prevalent respiratory involvement while 3 patients had a prevalent peripheral hypereosinophilia. Two patients had also cardiomyopathy and 1 had a eosinophilic gastritis; 6/7 patients had previously failed therapy with imatinib. *Results.* Five out seven patients achieved a response: 4/4 with respiratory involvement obtained a remission of the symptoms. Notably, one patient with chronic rhinitis also recovered from the associated anosmia after six infusions. 1/3 patients with peripheral hypereosinophilia showed a drastic eosinophil reduction after the first Mepolizumab administration. The other two patients did not show significant improvement. Duration of response was variable among patients, ranging from 4 to 16 weeks (mean 10.2). Notably, all patients re-achieved signs/symptoms remission when re-treated at relapse and, interestingly, clinical remission duration after maintenance courses was comparable, for each patients, to that recorded after induction. The mean number of maintenance cycles was 7.6 (range 3-17). With a median follow-up of 26 months (range 7-52) no patient has lost response. Neither Mepolizumab-related adverse events nor allergic reactions during infusion were reported. *Conclusions.* Though in a limited series, our study confirmed that Mepolizumab is safe and effective in controlling symptoms in HES patients. Notably, sensitivity to Mepolizumab was maintained after repeated cycles and no acquired resistance as well as late toxicity were recorded. Finally, response duration was variable among patient but, being consistent in each case, a patient-adjusted maintenance schedule might be used for symptoms recurrence prevention. *Aknowledgements.* BolognAIL, European LeukemiaNet, AIRC, Fondazione Del Monte di Bologna e Ravenna, FIRB 2006, PRIN 2008, Ateneo RFO, Project of Integrated Program (PIO), Programma di Ricerca Regione - Università 2007-2009.

0982

CLONAL MAST CELL DISORDERS LIMITED TO BONE MARROW: NOT A NEGLIGIBLE DISEASE

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Background. The diagnosis of mastocytosis in absence of the typical skin lesions is often difficult. It should be considered in the differential diagnosis of a number of unexplained clinical conditions, such as severe anaphylaxis, osteoporosis, neurological or constitutional symptoms, and chronic diarrhoea. Isolated bone marrow mastocytosis (BMM) is considered a rare subcategory of indolent systemic mastocytosis (ISM), characterized by low burden of mast cells (MC), absence of skin, lymph node or multiorgan involvement, slightly elevated serum tryptase (sT) levels, and a good prognosis. The term Monoclonal Mast cell Activation Syndrome (MMAS) is used to define those patients with mediator-related symptoms and absence of skin lesions in which bone marrow (BM) biopsy fails to demonstrate multifocal MC aggregates (i.e. the major criterion for diagnosing mastocytosis), but there is a proof of MC clonality (abnormal MC immunophenotype or detection of a KIT mutation). Data about these conditions are scanty. *Aims.* To define the frequency and characteristics of clonal MC disorders limited to BM in a cooperative multidisciplinary setting. *Methods.* From January 2006 to December 2009 144 adult patients with a clinical suspect of mastocytosis were referred to our multidisciplinary ambulatory for Mastocytosis, based on close collaboration between Hematologists, Allergists, Dermatologists and Rheumatologists. All patients underwent BM evaluation that included histology/citology, flow cytometry, and detection of KIT mutations, performed as described (Bonadonna *et al.*, JACI 2009).

Table. Characteristics of pts with clonal MC disorders.

	BMM	MMAS	ISM*	p#
N° of patients	40	13	48	
M/F	25/15	13/0	19/29	.001
Age at diagnosis (years)	49.5 (19-74)	51 (25-69)	42 (25-77)	ns
Time from first symptoms (months)	41.2 (2.9-365)	60.2 (9.3-164)	126.2 (0-480)	.001
Serum tryptase (ng/mL)	23.6 (10.6-108)	17.4 (12.7-27)	40.0 (7.7-761)	.001
% abnormal BM mast cells at flow cytometry (CD2+ and/or CD25+)	0.12 (0-0.78)	0.02 (0-0.11)	0.11 (0-2.3)	.007
Mediator-related symptoms (other than anaphylaxis)	10 (25%)	1 (7.7%)	20 (41.7%)	.03
Anaphylaxis	38 (95%)	13 (100%)	12 (25%)	<.0001
Allergy	11 (27.5%)	0	10 (20.8%)	ns
Loss of bone mineral density (osteopenia/osteoporosis)	8/33 (24.2%)	4/13 (30.7%)	10/30 (33.3%)	ns

* without skin involvement; # p value refers to BMM+MMAS vs ISM

Results. According to the current WHO guidelines, we made 111 diagnoses of clonal MC disorders: ISM with skin involvement were 48 (43.2%), BMM 40 (36%), MMAS 13 (11.7%). The remaining patients had Cutaneous Mastocytosis (9) and Mast Cell Leukemia (1). Among the 53 patients with clonal MC disorders limited to BM, the large majority of them (96%) had experienced one or more episodes of severe anaphylaxis after hymenoptera sting, drug or food or showed unexplained anaphylaxis. Two patients were referred to our Ambulatory because of unexplained osteoporosis. Compared to cases of ISM with skin involvement, patients with BMM or MMAS were predominantly males and, as expected, they had an inferior burden of MC, as suggested by the lower sT levels and the inferior percentage of BM abnormal MC at flow cytometry (see Table). Moreover, mediator-related symptoms other than anaphylaxis, such as pruritus, flushing, diarrhoea, hypotension, were less frequent in patients with MC disease limited to BM. Densitometric examination revealed 22/76 cases (29%) of osteoporosis or osteopenia, according to WHO definitions, without differences between subgroups. After a median follow-up of 22 months (range 1-48) all patients are alive and have stable disease. *Conclusions.* Clonal MC disorders limited to BM are a challenge for the physician. A close collaboration between different specialists may allow that more patients with clinical suspicion of mastocytosis could be referred to the opportune diagnostic procedures. In our experience this led to individuate 53 cases of isolated BMM or MMAS, the largest series reported to date. Besides making a correct diagnosis, this approach revealed a significant proportion of patients with loss of bone mineral density, so improving the management of this disease.

0983

PROGNOSTIC IMPLICATIONS OF THE EUROPEAN CONSENSUS FOR GRADING OF BONE MARROW FIBROSIS IN PRIMARY MYELOFIBROSIS: COMPARISON WITH THE PROGNOSTIC SCORING SYSTEM OF THE IWGMRT

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Background. The WHO classification proposes strict diagnostic criteria for primary myelofibrosis (PMF), and a European consensus has recommended the four-class grading of bone marrow fibrosis (BMF) as assessed by means of trephine bone marrow biopsies. We have previously generated a prognostic model for overall survival (OS) based on the WHO classification and the European consensus for BMF grading: in 98 consecutive PMF patients, our histological model significantly associated with differences in OS and, unlike other validated prognostic scoring systems (PSSs) (Mayo, Dingli, Cervantes, and Dupriez), clearly discriminated the OS of intermediate- and high-risk patients. The International Working Group for Myelofibrosis Research and Treatment (IWGMRT) has recently proposed a PSS for PMF that is structurally based on five clinical parameters predicting shortened survival: an age of >65 years, the presence of constitutional symptoms, hemoglobin levels of <10 g/dL, leukocyte counts of >25x10^9/L, and circulating blast cells ≥1%. These variables delineated four risk groups with distinct survival curves: low risk (0), intermediate risk-1 (1), intermediate risk-2 (2), and high risk (≥3). *Aims.* To establish the relationship between the BMF grading model and the IWGMRT PSS in PMF, and verify whether the fibrosis grading system helps to discriminate prognosis in patients with equal IWGMRT PSS scores. *Patients and methods.* The study involved 196 consecutive PMF patients (104 M, 96 F; median age 65.6 years, range 27-85 years), who were diagnosed between 1996 and 2008 (median follow-up 51.4 months, range 7.4-159 months), and classified using the WHO criteria. On the basis of the BMF grading model, 83 cases were classified as MF-0, 58 as MF-1, 41 as MF-2, and 14 as MF-3; on the basis of the IWGMRT PSS, 42 cases were classified as low risk, 73 as intermediate risk-1, 69 as intermediate risk-2, and 12 as high risk. OS curves were calculated according to Kaplan-Meier and compared by means of the log-rank test, and clinicopathological relationships were examined using the x2 test. A p value of <0.05 was considered significant. *Results.* OS was significantly shorter in the patients with MF-3 *vs.* MF-2 *vs.* MF-1 *vs.* MF-0 (Kaplan-Meier analysis, log-rank test: P<0.0001), and in the patients at high risk *vs.* intermediate risk-2 *vs.* intermediate risk-1 *vs.* low risk (Kaplan-Meier analysis, log-rank test: P<0.0001). The BMF grading model was capable of identifying at diagnosis patients at equal IWGMRT PSS risk with a different prognosis insofar as some of the patients with an equal risk score who died as a result of the disease had a higher degree of fibrosis. There was no good direct correlation between fibrosis grading and IWGMRT PSS scores, but the patients with lower MF values (0+1) more frequently had low or intermediate risk-1 IWGMRT PSS scores, and those with higher MF scores (2+3) more frequently had intermediate risk-2 or high risk IWGMRT PSS scores (x2 test: P<0.0001). *Conclusion.* The histological model significantly associated with differences in OS and could identify at diagnosis patients with the same IWGMRT PSS score but a shorter OS.

0984

GERMLINE HOMOZYGOSITY FOR JAK2 46/1 HAPLOTYPE IS A RISK FACTOR OF DEVELOPING PRIMARY OR POST-POLYCYTHEMIA VERA/ESSENTIAL THROMBOCYTHEMIA MYELOFIBROSIS

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Background. Recently a germline JAK2 haplotype (designated '46/1' or 'GGCC' haplotype) was described as a risk factor for acquiring JAK2 V617F-positive myeloproliferative neoplasms (MPN). The role of 46/1 haplotype as a predisposition factor for V617F-negative MPN is controversial. Previous studies have extensively investigated the relationship between the presence of acquired V617F mutation and the presentation of MPN. As carriership of 46/1 haplotype increases the risk of acquisition JAK2 V617F mutations, it may also have an impact on disease phenotype. *Aims of our study were the following.* (a) to examine and confirm associations of MPN and the presence of the JAK2 46/1 haplotype in Hungarian MPN patients; (b) to examine associations of the 46/1 haplotype with distinct clinical characteristics of MPN (especially the occurrence of complications affecting life expectancy such as thrombosis and myelofibrotic or leukaemic transformation). *Methods.* We genotyped 46/1 haplotype in 334 MPN-patients and 331 controls by a novel approach allowing the specific amplification of 617V and 617F alleles separately and genotype-detection of the rs12343867 tagging 46/1 haplotype. *Results.* The frequency of JAK2 V617F was 75.1% (251/334) in the MPN group: 87.4% (153/175) in polycytaemia vera (PV) patients, 59.5% (78/131) in essential thrombocythaemia (ET) patients, and 71.4% (20/28) in primary myelofibrosis (PMF) patients. In agreement with previous studies, increased frequency of allele 'C' linked to 46/1 was found among V617F-positive MPN-patients (n=251) compared to controls (44.3%±3.8% *vs.* 29.3%±3.5%, P<0.0001) and compared to V617F-negative MPN (31.3±7.2%; P=0.001). Excluding V617F-negative PV, the 'C' allele frequency was also increased among V617F-negative ET and PMF (n=61, 34.4%±8.6%, P=0.05) compared to controls. At presentation, significantly elevated hemoglobin (Hb) levels were found in V617F-positive patients compared to V617F-negative counterparts (P<0.000). Vascular complications were more common in V617F-positive patients (P=0.039, 26.6% *vs.* 15.2%). Although 46/1 haplotype is associated with the development of V617F mutation, neither hemoglobin level nor the occurrence of vascular complications were influenced by 46/1 genotype. Transformation to myelofibrosis was more frequent in CC-homozygotes (17/53, 32%) compared to patients with CT and TT genotypes (32/275, 12%; P<0.001) in the entire MPN-cohort. *Conclusions.* Our study confirms earlier observations that JAK2 46/1 haplotype is a susceptibility factor for JAK2 V617F-positive MPN and raises the possibility of a similar effect in JAK2 V617F-negative ET and PMF. The enrichment of 46/1 haplotype carriers among V617F negative MPN supports the 'fertile ground hypothesis'. 46/1 haplotype is not associated with MPN manifestations like disease type, splenomegaly, signs of increased erythropoiesis or myelopoiesis, vascular complication or leukaemic transformation except for the increased risk of the development of myelofibrosis in homozygous cases. In our hypothesis, clonal cells carrying 46/1 haplotype in homozygous form may be more susceptible to somatic recombination resulting in uniparental disomy (UPD) compared to 46/1 heterozygous cells, because of the perfect match between large portions (approx. 280 kb corresponding to the haplotype block) of the two parental 9p chromosome regions. Once UPD occurs, the affected clones may have proliferational advantage, therefore an increase of allele burden can be observed, which may be a natural evolution of MPN.

0985

DOWNREGULATION OF P53 BY HYDROXYUREA ASSOCIATED WITH A LACK OF APOPTOSIS INDUCTION IN CULTURED HUMAN KERATINOCYTES

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Hydroxyurea (HU) is commonly used for cytoreduction in patients with myeloproliferative neoplasms such as essential thrombocythaemia (ET). Nevertheless, this agent may have genotoxic and mutagenic properties and the possibility of long-term leukaemogenicity in patients with ET remains a serious safety concern. In addition, HU is associated with a variety of cutaneous side effects including skin atrophy, hyperpigmentation, alopecia, leg ulcers, actinic keratosis and, most seriously, squamous cell carcinoma. Importantly, HU-induced skin cancers may metastasize and are a potentially life-threatening complication. The tumour suppressor protein p53 is a transcriptional master regulator that protects against malignant transformation. By inducing cell cycle arrest and activating apoptotic pathways in response to DNA damage or other cellular stressors, p53 provides an important safeguard mechanism against malignant transformation. Inactivation of such pathways is thought to be a key mechanism of tumourigenesis. In order to investigate potential mechanisms linking HU to skin cancers, we investigated the effect of this agent on the apoptotic responses of cultured normal human keratinocytes and normal human melanocytes. Although HU treatment was associated with morphological changes and reduction in cell numbers in both cell types, there was a striking difference in the apoptotic response between melanocytes and keratinocytes. In melanocytes, HU led to the accumulation of p53, as demonstrated by Western blot analyses of total and nuclear protein extracts, and induced significant apoptosis starting around 48 hours post-treatment. Functional p53 was assayed by its DNA binding capacity. In contrast, there was only limited induction of apoptosis by HU in keratinocytes, which was correlated with p53 downregulation at 24 hours and 48 hours post-treatment. HU exerted significant damage on melanocytes and keratinocytes, but it is the downregulation of the tumour suppressor protein p53 in surviving keratinocytes which could provide a mechanistic rationale for the occurrence of skin cancers in ET patients treated with HU.

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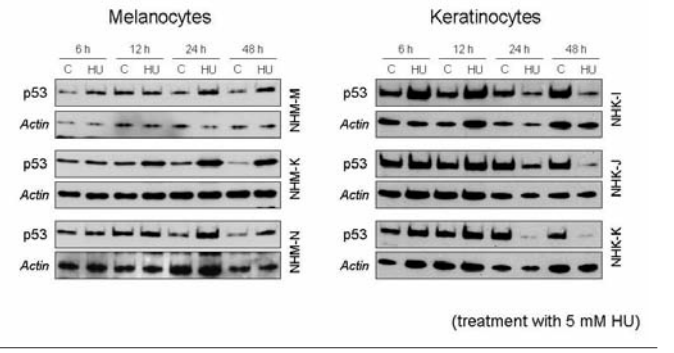

Figure. Nuclear extracts: regulation of p53.

0986

SEVEN YEAR FOLLOW-UP SHOWS THAT WHO BONE MARROW CRITERIA EFFECTIVELY IDENTIFIES TRUE ET WITH NO FIBROSIS

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Background. At present, the usefulness of the new World Health Organisation (WHO) classification bone marrow criteria for MPN (myeloproliferative neoplasms) is under debate. A high incidence of clinical manifestations of myelofibrosis in a cohort of patients with essential thrombocythemia (ET) and polycythemia vera (PV) initiated a blinded re-evaluation of diagnosis with the use of the new WHO bone marrow criteria. *Aims.* To examine the development of bone marrow fibrosis in patients with ET and PV followed for a minimum of 7 years. In addition, to re-evaluate the diagnosis of ET based on old criteria, using the WHO classification. *Methods.* This prospective study of 60 patients with newly diagnosed or previously treated MPN, (42 ET, 17 PV and 1 PMF), was set up in 1998 to evaluate platelet reduction treatment. Informed consent was obtained. Two-year follow-up results

are previously reported. Bone marrow trephine biopsy specimens were requested from patients at study start, after 2 and 7 years of follow-up. The biopsies were assessed jointly by two experienced hematopathologists (HK, JT). The examination was blinded, without knowledge of time of sampling or any clinical data, except age and sex. WHO criteria were used for diagnosis and fibrosis grading. *Results.* At 7 years 2 patients had transformed to MDS, 2 to AML, 7 to myelofibrosis (WHO 2008 criteria). Ten patients were dead, 2 with PV, 2 with true ET (one trauma, one respiratory insufficiency), 6 with PMF. One was lost-to-follow-up. Of the 42 patients with initial diagnosis ET, there were bone marrow specimens of good quality from 40 patients at study start. The blinded bone marrow analysis identified 21 of the 42 initial ET patients as true ET (WHO criteria). 19 were alive, and in the 12 with available follow-up biopsies none had fibrosis. 15/21 had an available JAK2V617F test, 8 were positive (53%). Out of the 42 patients with initial diagnosis ET, 17 were reclassified as PMF (12 PMF-0, 3 PMF-1, 2 PMF-2), 2 as MPN-U. JAK2V617F results were available in 10/17 with PMF, only 1 was positive (P= 0.034 *vs.* ET). In the PMF cases, fibrosis progressed: 9 had a fibrosis grade of 2 or higher at study end, 7 of these patients had a WHO-compatible PMF. In PV patients, representative bone marrow specimens from study start were available from 15/17. Three of the PV-patients had fibrosis grade 1. At 7-year follow up, from the 14 patients still alive, we received 10 representative bone marrow samples showing that 2 patients had grade 1 fibrosis and 3 grade 2. JAK2 results were available in 11 PV patients, 10/11 were positive. *Conclusions.* A high incidence of bone marrow fibrosis in a cohort of ET and PV patients diagnosed before 1998 was explained by re-evaluation of the initial ET diagnosis with the use of the new WHO criteria. These criteria were able to differ blindly between true ET and PMF with a marked difference in follow-up outcome. Relations between fibrosis development and treatments are under analysis and will be reported separately.

0987

TIME-DEPENDENT ANALYSIS IN PATIENTS WITH PRIMARY MYELOFIBROSIS SHOWS THAT RED BLOOD CELL TRANSFUSION DEPENDENCY AFFECTS SURVIVAL AND IMPROVES DYNAMIC RISK STRATIFICATION

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Background. Primary Myelofibrosis (PMF) is a Philadelphia-negative myeloproliferative neoplasm (MPN) with a heterogeneous clinical presentation, encompassing anemia, splenomegaly, leukocytosis or leukopenia, thrombocytosis or thrombocytopenia, and constitutional symptoms. Current risk stratification is based on the International Prognostic Scoring System (Cervantes *et al.*, Blood 2009), developed to predict survival at diagnosis, (IPSS) and on dynamic IPSS (DIPSS), to be applied anytime during follow-up (Passamonti *et al.*, Blood 2010). Both models do not take into account the potential impact on survival of red blood cell (RBC) transfusion-dependency, which may be present at diagnosis or acquired during follow-up. *Aim.* To investigate whether RBC transfusion-dependency (at diagnosis or dynamically acquired) affects survival of PMF patients. *Methods.* This study includes 296 consecutive patients with PMF diagnosed between 1980 and 2009 and followed at the Division of Hematology, IRCCS Fondazione Policlinico San Matteo, University of Pavia. This study was in accordance with the Helsinki Declaration of 1975. Diagnosis of PMF was based on the presence of megakaryocyte proliferation and atypia accompanied by increased reticulin and/or collagen in bone marrow as major criterion, as well as of the JAK2 (V617F) or the MPL mutations if available and two criteria among anemia, splenomegaly, increased lactate dehydrogenase level, and leukoerythroblastosis. Transfusion-dependency was defined as the persistence of RBC transfusion need to correct anemia (Hb <8.5 g/dL). The effect of RBC transfusion-dependency on survival was evaluated by Cox proportional hazards regression with time-dependent covariates on 226 patients with a regular follow-up. *Results.* Median follow-up was 3.3 years (range, 0-18) with 1401 persons/year of follow-up. Median age at diagnosis was 61 years (range, 24-86) and male/female ratio was 193/103. IPSS risk stratification at diagnosis (n=263) grouped patients as low (77, 29%), intermediate-1 (70, 27%), intermediate-2 (73, 28%) and high risk (43, 16%). RBC transfusion-dependency was present at diagnosis in 44 (15%) of 296 patients. Kaplan Meyer analysis showed that RBC transfusion-dependency significantly affects survival (P<0.001). Median survival was 2.6 years in patients who received RBC transfusion at diagnosis and 8.1 years in those who did not. After adjusting for IPSS categories in multivariable Cox proportional hazard regression, transfusion-dependency retained a significant impact on survival (HR 1.71, 95%CI: 1.07-2.75; P=0.03). During follow-up, 39 (17%) of 226 regularly followed patients became RBC transfusion-dependent. To investigate the dynamic impact of RBC transfusion-dependency on survival, a univariable analysis by Cox regression using transfusion status as time-dependent covariate was carried out. We found that patients who acquire RBC transfusion-dependency anytime during follow-up have a significantly worse survival compared to those who remain transfusion-independent (HR: 5.06, 95%CI: 3.36-7.63; P<0.001). In a multivariable analysis with DIPSS categories and transfusion status as time-dependent covariates, RBC transfusion-dependency retained its prognostic impact on survival (HR 3.69, 95%CI: 2.43-5.6; P<0.001).*Conclusions.* This study indicates that RBC transfusion-dependency, either present at diagnosis or acquired anytime during follow-up, is a significant prognostic factor for survival in patients with primary myelofibrosis. Transfusion status results as a useful complement to IPSS and to DIPSS.

0988

EXTRA-HAEMATOLOGICAL ADVERSE EVENTS IN ESSENTIAL THROMBOCYTHAEMIA PATIENTS TREATED WITH HYDROXYUREA: A PRELIMINARY REPORT OF THE REGISTRO ITALIANO TROMBOCITEMIA (RIT)

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Background. Hydroxyurea (HU) is the cytoreductive drug more frequently used for the first-line treatment of Essential Thrombocythemia (ET) patients. The HU efficacy in reducing the rate of thrombotic complications is well documented, and its use is limited only by the potential leukaemogenicity and by the haematological and extra-haematological toxicity and side effects. *Objective.* To evaluate the extra-haematological toxicity/side effects of HU in a large series of ET patients. *Material and methods.* One thousand and seventy-five ET patients of the RIT treated with HU are the object of this report. The patients, 641 females (59.6%) and 434 males (40.4%), 2-93 years old (median age 71), diagnosed according to the PVSG or WHO criteria, received HU as first or second line treatment in seventy haematological centres. The administered dose of HU was 0.25-3.0 g/day, with a median value of 1g/day; the HU dose was 1.5-2.0 g/day in 75 cases (7.0 %) and >/= 2 g/day in 33 cases (3.1%). The following adverse events (AE) reported as related to the HU treatment were considered for the analysis: dermatological (hyperpigmentation, rash, lichen, carcinoma, leg ulcers); gastro-intestinal (nausea/vomiting, diarrhoea); general (fever, myalgia); other. Moreover, the causes of HU withdrawal were analysed. *Results.* During the follow-up, in the ET patients treated with HU, the reported dermatological AE were: hyperpigmentation 41 cases (3.8%), rash 27 cases (2.5%), lichen 13 cases (1.2%), carcinoma 8 cases (0.7%), leg ulcers 30 cases (2.8%); the gastro-intestinal AE were: nausea/vomiting 42 cases (3.9%), diarrhea 36 cases (3.3%); the general AE were: fever 34 cases (3.2%), myalgia 57 cases (5.3%); the other AE were 88 cases (8.2%). The HU withdrawal referred to extra-haematological AE were 56 cases (5.2%). By distinguishing the ET patients diagnosed before the year 2004 (432, 40.2%) and diagnosed since 2004 (642, 59.7%), the rate of all subgroups of extra-haematological AE was appreciably reduced in the patients diagnosed more recently. *Conclusion.* This preliminary analysis shows that the extra-haematological AE referred to the HU treatment in the ET patients of the RIT were not negligible and allowed to the drug withdrawal in more than 5% of cases.

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0989

IN PATIENTS WITH ESSENTIAL THROMBOCYTHEMIA, LEUKOCYTOSIS AT DIAGNOSIS IS A NEGATIVE PROGNOSTIC FACTOR FOR PROGRESSION TO MYELOFIBROSIS

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Background. About half patients with Essential Thrombocythemia (ET) carry the JAK2V617F mutation; they rarely progress to myelofibro-

sis (MF)(rr 4% at 15 years). At present, no simple test is available to expect the progress to MF (PET-MF). As an example, no meaningful difference between JAK2V617F mutated and non mutated patients has been found in the respect of PET-MF, even if the accumulation of mutant V617F alleles has been reported to be a mechanism of evolution toward MF in JAK2V617F mutated ET patients. *Patients and methods.* We evaluated retrospectively a cohort of 146 patients affected by ET (M 35, F 111, median follow up 10.46 y, mean age at diagnosis 51.2±17.2 y) diagnosed in agreement with WHO criteria. We compared the white blood cells (WBC) number at diagnosis of 13 patients progressed to MF (PET-MF) with133 cases who did not evolve (ET). Considering that the patients with ET had very wide durations of their follow-up, we selected within this cohort, 20 patients (ET-2) who were comparable for sex, age and follow-up duration to PET-MF patients. The comparison between means was performed with One-way ANOVA and the threshold of WBC number has been defined with ROC curve. *Results.* The main hemochrome data and mutational status of patients are summarized in Table 1. WBC counts at diagnosis were statistically higher in PET-MF than in ET and ET-2 (P=0.0001). The threshold WBC count significant for MF progression was identified at 10.2×10^9/L (sensibility 83%, specificity 81%) comparing group PET-MF with ET and at 9×10^9/L (sensibility 80%, specificity 92%) comparing with ET-2. No statistical differences were found in mean platelet counts and in mean hemoglobin levels. *Discussion.* As expected, most PET-ET patients were JAK2V617F. This study shows that an increased number of WBC at diagnosis is a relevant marker of MF progression in patients with ET. In contrast, the contribution of Hb level and platelets number at diagnosis to MF progression remains not significant. We suggest to monitor JAK2 allele burden mainly in patients with ET and a high WBC number at diagnosis.

Table 1.

	WBC x 10^9/L	Hemoglobin (Hb) g/L	Platelets (Plts) x 10^9/L	JAK2 V617F MT/WT
ET	8.52 ± 2.65	13,76 ± 1,40	781,8 ± 273,8	67/42
PET-MF	13.7 ± 6.3	14,6 ± 1,3	780,7± 366,1	8/4
ET-2	8.2 ± 2.8	14,2 ± 1,17	694.6 ± 158.7	14/6

0990

INCREASED RISK OF SPLANCHNIC OR CEREBRAL VENOUS THROMBOSIS IN PATIENTS WITH ESSENTIAL THROMBOCYTHEMIA CARRYING THE JAK2 V617F MUTATION

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Background. We previously reported in a cohort of patients with splanchnic venous thrombosis (SVT) or cerebral venous thrombosis (CVT) the presence of the JAK2 V617F mutation in 95% of patients with an overt Philadelphia-negative myeloproliferative neoplasm (MPN), in 21% of patients with SVT and without overt MPN, and in 5% of patients with CVT and without overt MPN (De Stefano *et al.*, J Thromb Haemost 2007; 5: 708). Moreover, the mutation is actually known to be associated with an increased risk of thrombosis in patients with essential thrombocythemia (ET). *Aims.* To investigate the impact of the JAK2 V617F mutation on the risk of thrombosis in unusual sites in patients with ET. *Patients and ethods.* We carried out a retrospective cohort study on 224 patients with ET (M/F 75/149, median age at diagnosis 55 years, range 20-92): 156 were asymptomatic and the remaining ones had suffered from arterial thrombosis (n=39,17.4%), venous thrombosis in common sites (n=13, 5.8%), SVT (n=13, 5.8%), and CVT (n=3, 1.3%). All the patients were tested for the presence of the JAK2 V617F mutation and inherited thrombophilia. *Results.* The mutation was present in 144 patients (64.2%), namely in 87 asymptomatic ones, in 41 with arterial thrombosis or venous thrombosis in common sites, in 11 with SVT, and in all the three patients with CVT. Therefore, in ET patients the relative risk (RR) of thrombosis in unusual sites (SVT or CVT) associated with the mutation was 1.56 (95%CI 1.24-1.97) in comparison with the patients without history of thrombosis, namely 1.51 (95%CI 1.15-1.98) for SVT, and 1.79 (95%CI 1.55-2.06) for CVT. All the patients with SVT or CVT were aged <60 years: among the patients within this age range, the RR associated with the mutation in respect to the asymptomatic individuals was 1.81 (95%CI 1.36-2.41) for overall thromboses in unusual sites, 1.75 (95%CI 1.27-2.40) for SVT, and 2.07 (95%CI 1.66-2.57) for CVT. Those results did not substantially change after exclusion of the patients with inherited thrombophilia: one with SVT, one with CVT, and five without thrombosis (data not shown). Notably, in the patient cohort the mutation did not increase the risk of venous thrombosis in common sites (RR 1.24, 95%CI 0.84-1.83). *Conclusions.* In ET the risk for venous thrombosis associated with the JAK2 V617F mutation preferentially targets splanchnic or cerebral veins in young patients, and is independent of inherited thrombophilia.

0991

ANAGRELIDE AND FIBROBLAST GROWTH FACTOR-2 LEVELS IN PATIENTS WITH ESSENTIAL THROMBOCYTHEMIA

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Background. Essential thrombocythemia (ET) is characterized by platelet-coagulant-endothelial and angiogenic activation. Fibroblast growth factor-2 (FGF-2) is released by platelet-endothelial activation and upregulated by fibrinogen (Fg). It has been reported that FGF-2 induces myeloproliferation. The standard treatment includes cytoreduction with hydroxyurea (HU) or anagrelide (ANA) and antiplatelets. *Aims.* We evaluated platelets, platelet factor 4 (PF4) and fibrinogen (Fg), as markers of platelet and coagulant activation, tissue factor pathway inhibitor (TFPI) and von Willebrand factor (vWF), as markers of endothelial activation, FGF-2 and vascular endothelial growth factor (VEGF), as indicators of angiogenesis, and white blood cell (WBC) count, as myeloproliferative index. *Methods.* We recruited 42 patients with ET (21 males and 21 females, mean 60 years) who fulfilled WHO criteria. Their mean duration of disease was 8 years (range, 4-21 years). None of patients had splenomegaly. Of 42 patients, 21 were on HU and 21 were on ANA. The average dose of HU was 1.25 g/day. ANA was administered in dose of 0.5 mg/day, with increases of 0.5 mg/day every 7 days until the platelets decreased below 400×10^9/L and with a average maintenance dosage of 2.1 mg/day. All patients were on aspirin. Platelets, PF4, Fg, TFPI, vWF, FGF-2, VEGF and WBC were measured before cytoreduction and to complete response defined as platelets $<400\times10^9$/L. Platelets and WBC were measured by automated analyser. PF4, TFPI, FGF-2 and VEGF and Fg and vWF were assayed by ELISA and Clauss and immunoturbidimetric assay, respectively. Considering that FGF and VEGF may be produced by platelets, we adjiusted FGF and VEGF per platelet (FGFPLT /106 and VEGFPLT/106). *Results.* Before treatment all patients had thrombocytosis ($966\pm286\times10^9$/L), high PF4 (167±100 Iu/mL *vs.* 4.1±2.4 Iu/mL) (P<.0001) and Fg (388±96 mg% *vs.* 237±42 mg%) (P<.0001), elevated TFPI (130±71 ng/mL *vs.* 94±10 ng/mL) (P<.0001) and low vWF (26±9 % *vs.* 77±18%) (P<.0001), elevated FGF-2PLT (0,06±0.06 pg/106 *vs.* 0.01±0.001 pg/106) (P<.0001) and VEGFPLT (1.6±1.0 pg/106 *vs.* 0.5±0,5 pg/106) (P<.0001), leukocytosis ($9.3\pm2.1\times10^9$/L *vs.* $5.2\pm1.1\times10^9$/L) (P<.0001). After treatment, all patients had platelets $<400\times10^9$/L ($385\pm55\times10^9$/L) and normal WBC ($7.3\pm2\times10^9$/L). PF4, Fg, TFPI, FGF-2PLT and VEGFPLT remained elevated (190±90 Iu/mL *vs.* 4.1±2.4 Iu/mL and 465±140 mg% *vs.* 237±42 mg% and 138±39 ng/mL *vs.* 94±10 ng/mL and 0.13±0.13 pg/106 *vs.* 0.01±0.001 pg/106 and 1.5±1 pg/106 *vs.* 0.5±0,5 pg/106) (P<.0001 and P<.0001 and P<.0001 and P<.0001 and P<.0001 and P<.0001, respectively) and vWF was low (33±7 % *vs.* 77±18%) (P<.0001) in the HU group whereas the ANA group normalized PF4 (8.5±3 Iu/mL), Fg (295±46 mg%), TFPI (100±49 ng/mL), vWF (91±35 %), FGF-2PLT (0.01±0.0 pg/106) and VEGFPLT (1.1±0.6 pg/106). A positive correlation there was between PF4 and platelets and Fg and TFPI and vWF (P=0.042 and P<.0001 and P=0.067 and P<.0001, respectively) and FGF-2PLT (P<.0001) and between FGF-2PLT and Fg (P<.0001). A significant correlation there was between FGF-2PLT and TFPI and vWF (P=0.011 and P<.0001, respectively). A correlation was found between FGF-2PLT and VEGFPLT (P<.0001) and WBC (P=0.008). *Conclusions.* These data suggest that FGF-2PLT may be a platelet-coagulant-endothelial-activation thrombotic marker that normalizes after ANA ameliorating the prognosis of ET.

Platelet biology

0992

A NOVEL SPLICE-DONOR SITE MUTATION IN THE GPIIIA GENE IDENTIFIED IN FOUR WOMEN WITH REDUCED FIBRINOGEN RECEPTOR EXPRESSION

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Background. The fibrinogen receptor GPIIb-GPIIIa is the most abundant platelet receptor and crucial for the clotting process. Mutations in the genes coding for the GPIIb-GPIIIa complex result in qualitative and/or quantitative changes of the receptor and is the molecular background for the bleeding disorder Glanzmann thrombasthenia (GT). Two different genes encode the two subunits _II_ (GPIIb) and _3 (GPIIIa) which are associated by non-covalent and calcium dependent bonds. Complex formation is imperative for surface expression. The human platelet antigen-1 (HPA-1) is defined by the SNP T176C in the GPIIIa gene, resulting in a Leu33Pro polymorphism giving rise to the antigens HPA-1a and HPA1b, respectively, in the mature GPIIIa protein. We have identified four women who were phenotypically HPA-1a negative whereas genotyping showed that they were HPA1-ab. Flow cytometry studies revealed that the expression of the fibrinogen receptor was reduced by approximately fifty percent, indicating that they might be carriers of the recessive hereditary bleeding disorder GT. *Aims.* We sought to delineate the molecular background for the genotype/phenotype discrepancy and reduced fibrinogen receptor expression. *Methods.* DNA and cDNA sequencing were used to identify possible mutations, whereas expression vector cloning in CHO cells was used for fibrinogen receptor expression analysis. *Results.* DNA sequencing revealed a previously not described intron mutation in GPIIIa, IVS6(+4)A>G, heterozygously present in all four women. Sequencing of GPIIb showed no mutations. cDNA sequencing of the women's platelet mRNA indicated that exon 6 was missing. Subsequent cloning of the PCR fragments showed that exon 6 was lacking in several transcripts. Co-tranfection of the GPIIb subunit with GPIIIa as wild type or lacking exon 6 revealed that the GPIIb-GPIIIa complex with GPIIIa lacking exon 6 hardly had any surface expression compared to wild type. *Conclusion.* The novel splice-donor site mutation identified in the GPIIIa gene show skipping of exon 6 and a fifty percent reduction of the fibrinogen receptor expression in four women who had the mutation in heterozygous form.

0993

RAPID EXCLUSION OR CONFIRMATION OF HEPARIN-INDUCED THROMBOCYTOPENIA: A SINGLE-CENTRE EXPERIENCE WITH 1291 PATIENTS

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Background. Untreated heparin-induced thrombocytopenia (HIT) may lead to lethal thrombotic events and administration of alternative anticoagulants may be complicated by severe bleeding. It is therefore most desirable to confirm or exclude HIT as soon as its diagnosis is suspected. *Aim.* To evaluate the negative (NPV) and positive (PPV) predictive values of the 4T clinical score and three immunoassays for the presence of platelet-activating HIT-antibodies (abs). *Methods.* Assessment of 4T score, semi-quantitative result (titer) of rapid ID-HPF4-PaGIA, optical densities (OD) of two commercial immunoassays (HPIA, GTI-PF4) and heparin-induced platelet aggregation test (HiPAT) in 1291 patients investigated for suspected HIT. A positive HiPAT was the gold-standard for *in vitro* platelet-activating HIT-abs. *Results.* Among 859 patients with a low clinical probability for HIT (4T score = 0-3), 7 had a positive HiPAT, resulting in a NPV of 99.2%. Fifty out of 358 patients with an intermediate 4T score (4-5), and 39/74 patients with an high 4T score (6-8) had a positive HiPAT, resulting in PPV of 14.0% and 52.7%, respectively. Among laboratory assays, the rapid ID-HPF4-PaGIA and both ELISAs performed similarly, with AUC of ROC-curves of 0.992 (Titer), 0.990 (OD HPIA) and 0.985 (OD GTI-PF4). All three assays were able to exclude the presence of platelet-activating HIT-abs (ID-HPF4-PaGIA titer ≤1, HPIA OD <0.300, GTI-PF4 OD <0.870). The cut-offs with the best compromise between sensitivity and specificity for functionally relevant HIT-abs were a titer of ≥4 (ID-HPF4-PaGIA), OD >0.943 (HPIA) and OD >1.367 (GTI-PF4). Most interestingly, only the ID-HPF4-PaGIA showed a PPV of 100% for a positive HiPAT, at titers of ≥32. *Conclusions.* HIT can neither be diagnosed nor refuted on clinical grounds alone. A negative immunoassay among patients with low and intermediate 4T scores makes HIT very unlikely. Of note, a titer of ≥32 by ID-HPF4-PaGIA has a PPV of 100% for *in vitro* platelet-activating HIT-abs. This is the first report showing that the quantitative result of an immunoassay is equivalent to a positive functional assay for HIT-abs. A high-titer positive ID-HPF4-PaGIA allows for rapid laboratory confirmation within a few hours after HIT has been suspected.

0994

FCGRIIB RECEPTOR IS NOT REQUIRED FOR THE THERAPEUTIC ACTION OF IVIG IN AN ITP MOUSE MODEL

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Background. One of most recognized theories of therapeutic action of IVIg on immune thrombocytopenia (ITP) suggests that up-regulation and activation of Fc_RIIB in the spleen following IVIg treatment is critical to provide negative regulation of platelet clearance via decreased phagocytosis of opsonized platelets by mononuclear phagocytic cells in the spleen. We have not been able to support these previous findings but instead find the presence of Fc_RIIB receptor not necessary for successful treatment of experimental ITP through IVIg administration. *Methods.* Mice were purchased from Taconic (Germantown, NY, USA) and Jackson Labs (Bar Harbor, USA). To induce ITP in mice, daily administration of an escalating dose of antiplatelet antibody (anti-CD41; MWReg30) was used. IVIg (1-2 g/kg; Gammagard 5%) was obtained from Baxter Corporation (Toronto, ON, Canada). Blood samples were taken daily and platelet counts monitored by FACS. *Results.* Administration of IVIg to wild-type Balb/c mice previously made thrombocytopenic with antiplatelet antibody leads to amelioration of experimental ITP while in untreated mice platelet counts stay close to nadir. Similar dynamics were found using FcγRIIB knockout Balb/c mice despite the absence of FcγRIIB. However, as previously published, IVIg does not work with FcγRIIB knockout mice on a B6 background obtained from the Jackson Labs. Indeed, B6 (129S4-Fcgr2btm1Rav/J) FcγRIIB-/- knockout mice made thrombocytopenic are essentially unresponsive to IVIg treatment. However, surprisingly, we found that when using the proper control wild-type mice for this knockout, the B6.129SF2 mouse strain, we found that this wild-type FcγRIIB+/+ mouse also does not respond to IVIg treatment. Confirmation of genotype was done; thus, we suggest that something about the 129S4 background prevents a response to IVIg therapy and this phenomenon is independent of the FcγRIIB. Previous publications that indicated a lack of response to IVIG using the Jackson Labs FcγRIIB knockout mice failed to use the proper control animals, using instead, C57BL/6 wild-type mice which respond well to IVIg treatment. It was also reported that the protective effect of IVIg was associated with its ability to induce surface expression of FcγRIIB on splenic macrophages. However, our data showed that splenectomy does not affect the ability of IVIg to ameliorate ITP and there is no upregulation of FcγRIIB mRNA in the spleen following IVIg treaatment. *Conclusions.* Our results using Balb/c FcγRIIB knockout and splenectomized mice indicate that FcγRIIB is not a factor in whether or not IVIg treatment will be effective in ITP. Our results are consistent with a single previous report showing that Balb/c mice lacking FcγRIIB respond to IVIg treatment. Furthermore, our results provide an explanation for the discrepant published results using the Jackson mice by showing that the wild-type conrol animals for the B6 FcγRIIB knockout mice also do not respond to IVIg treatment; thus, the lack of response of the knockout animal is unrelated to FcγRIIB but to other factors related to its derivation. These data taken together lead us to the conclusion that the presence of the FcγRIIB receptor is not important for the therapeutic action of IVIg.

0995

BERNARD SOULIER SYNDROME IN A PATIENT AFFECTED BY KLINEFELTER DISEASE: NOVEL A386G HOMOZYGOUS MUTATION OF GPIBα GENE WITH ANOMALOUS BEHAVIOUR

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Background. Bernard Soulier Syndrome (BSS) is a rare inherited form of macrothrombocytopenia characterized by defective platelet membrane von Willebrand Factor (vWF) receptor. This complex is made of 4 subunits (glycoproteins (GPs) Ibα, Ibβ, IX, and V) belonging to the Leucine Rich Repeats (LRR) proteins family. Mutations of these GPs have been recognized as responsible of different forms of classical (recessive) or autosomal dominant BSS. In particular, many different gene mutations affect GPIbα, the major component, leading to amino acid substitutions or stop codons, often occurring after a frameshift producing aberrant protein sequences. *Aims.* In the present work we present the characterization of a new homozygous mutation of GPIbα gene in a patient affected by Klinefelter Syndrome. Moreover we had the opportunity to observe how the absence of vWF receptor on the platelet surface influences the megakaryopoiesis process. *Methods.* A 32-year-old man affected by Klinefelter syndrome and initially diagnosed as affected by idiopatic trombocytopenic purpura, was investigated for inherited BSS with functional studies (RIPA: Ristocetin agglutination), flow cytometry, Megakaryocytes cultures (MK), molecular biology tests (Sequencing, Western Blot, RTPCR) and computer modelling techniques. The patient gave his informed consensus to the study. *Results.* RIPA was absent, as is typical in BSS subjects. Flow cytometry and Western blot (WB) analysis showed the absence of GPIbα in patient's platelets. Sequencing results revealed a homozygous substitution A386G affecting the first base of the DNA codon codifying for N110 of GPIbα. This result do not explain the absence of the protein. In order to clarify flow cytometry and WB data, megakaryocytes (MK) cultures from patient and healthy control blood were set for further analysis. Although patient's MK morphology and maturation profiles were normal, immunofluorescence on cultured cells confirmed the absence of the protein Moreover, in the patient cultures proplatelets formation resulted defective, and GPIbα mRNA was absent Computational analysis of the mutant mRNA showed that the A386G mutation disrupt an ESE motif (Exon Splicing Enhancer) which can be involved in the correct maturation of the GPIba transcript, explaining the lack of the protein. *Conclusions.* This is, to our knowledge, the first report of BSS affecting a Klinefelter subject. The homozygous A386G substitution found in GPIbα gene of this patient, has an anomalous behaviour: theoretically it would be supposed to change the residue N110 of the protein in D. On the contrary, it cause the disappearing of the GPIbα protein without creating a stop codon in mRNA sequence, but disrupting an ESE motif and hypothetically interfering with the transcript correct maturation. Further analysis will be needed to confirm this hypothesis. Moreover, we could confirm with MK culture studies that the presence of the vWF receptor on MK surface is mandatory for proplatelets formation.

0996

PRIMARY BONE MARROW FIBROSIS IN INMUNE THROMBOCYTOPENIC PURPURA

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Bone marrow fibrosis in ITP has become a matter of discussion since the introduction of thrombopoietin receptor-stimulating agents. It is usually recommended to perform a bone marrow (BM) biopsy before the administration of these drugs in patients with ITP. Prospective studies have been conducted in this sense to investigate the presence of fibrosis after the administration of Romiplostin.[1] The incidence of marrow fibrosis in patients with ITP is unknown, mainly because clinical guidelines do not recommend a routine bone marrow biopsy in most of the patients with this disease. In order to determine the incidence of BM fibrosis in ITP, we have reviewed 175 bone marrow samples from patients diagnosed with ITP in our Unit in the past ten years. A bone marrow biopsy was obtained at diagnosis in 28 of them. Eight were women and seven men, age range: 16 - 89 years (mean 56.2). Bone marrow samples had been included in paraffin. For this review, new specimens were sliced and stained with hematoxylin-eosin and reticulin techniques. Samples were analysed by the Hemathopathologist of our Center. Assessment of fibrosis has been made according to the WHO criteria as follows: Grade 0 (scattered linear reticulin with no intersections (cross-overs), corresponding to normal bone marrow, Grade 1 (loose network of reticulin with intersections, especially in perivascular areas), Grade 2 (diffuse and dense increase in reticulin with extensive intersections, ocasionally with focal bundles of collagen and/or focal osteosclerosis) Grade 3 (Diffuse and dense increase in reticulin with extensive intersections and coarse bundles of collagen, often associated with osteosclerosis). Fibrosis was found in the bone marrow samples of 53% of the patients. Most of them showed Grades 1 to 2 (14 patients). Only one patient showed grade 3. A review of the medical records of that patients confirmed the diagnosis of ITP, excluding primary myelofibrosis. We conclude that fibrosis can be found in patients with ITP who have not been treated previously with thrombopoietin receptor-stimulating agents. The role of megakaryocytes, which are usually increased in the bone marrow of ITP patients, in the pathogenesis of the fibrosis may be a matter of future investigations.

Reference

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0997

CONGENITAL AMEGAKARYOCYTIC THROMBOCYTOPENIA (CAMT): A DIFFICULT EARLY DIAGNOSIS

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Background. CAMT is an extremely rare autossomal recessive disorder characterized by absent or reduced number of megakaryocites in the bone marrow (BM) since birth. Mutations in the c-MPL gen, coding for the Thrombopoietin (TPO) receptor, are responsible for CAMT. Children develop a tri-linear marrow aplasia that is fatal without BM transplantation. Therefore, an early diagnosis is recommended in order to minimize morbility and mortality of the aplasia. Historically, the evaluation of children with persistent thrombocytopenia has relied on serial bone marrow aspirates and biopsies. However, recently, the diagnosis can be confirmed by the demonstration of mutations in cMPL gen. *Aims.* to describe the clinical and molecular findings of a new case of CAMT. *Case report.* a Caucasian 2-year-old girl who presented with petechiae and ecchymosis, with a platelet count of 29.000/mcL. White blood cells count and hemoglobin were normal. She did not presented any bone alteration. However, she had a special phenotype geneticall ly not filiated (short stature and low weight, microcephaly, unilateral ptosis and low hair implant) and mild psychomotor retardation. Parents were not consanguineous. Alloimmune and autoimmune thrombocytopenia, immunodeficiency and serologies evaluation were negative. The first BM aspirate was normal. Cytogenetic was also normal and DNA breakage studies were negative. Fetal hemoglobin was 8%. In the next three months platelet count was around 30×10^9/L without any mucosal bleeding. A second aspirate and biopsy were performed. It revealed a reduced number of megakaryocytes. The study of growth hematopoietic progenitors showed an impaired growth of megakaryocytic progenitors. The TPO level was 2100 pg/mL (normal <168 pg/mL). We had already the clinical diagnosis of CAMT. 4 years later, cMPL gene´s mutations were investigated with these *Results.* a heterozygous nonsense mutation in exon 7 (maternal allele) and a heterozygous silent mutation in exon 4 (paternal allele). Currently, she is 12 years old. The platelet count is around 10×10^9/L, hemoglobin level 9 g/dL, neutrophil count 1×10^9 /L. She has been treated with corticotherapy and platelet transfusion due to parvovirus B19 infectious. She is waiting for an unrelated matched BM donor. *Conclusions.* regardless of the presence of megakaryocytes on early BM aspirates, if there is on-going thrombocytopenia that can not be attributed to another etiology, CAMT should be considered. Supportive evidence may

include an elevated hemoglobin F and levels of TPO nearly 30 times higher than healthy children. As aspirates can not definitively assess BM architecture or cellularity, a biopsy is required. Our case shows two different mutations, one a silent mutation. Since CAMT is recessively inherited, these findings are not sufficient to confirm the molecular diagnosis of CAMT. Nevertheless, mutations in the MPL gene are so rare in healthy donors, that a coincident occurrence of a MPL mutation in a patient with symptoms of CAMT is very unlikely. Probably, this fact could explain the late onset and the slow development to pancytopenia. Once the diagnosis is established, a search for a HLA-related/unrelated matched BM donor should be started.

0998

REDUCED EXPRESSION OF TRANFORMING GROWTH FACTOR–β1 AND CORRELATIVE ELEVATION OF INTERLEUKIN-17 AND INTERFERON-G IN PEDIATRIC PATIENTS WITH CHRONIC IMMUNE THROMBOCYTOPENIC PUPURA

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Background. Dysregulated T helper (Th) cells are considered important in the pathophysiology of chronic immune thrombocytopenic purpura (ITP). Although there was evidence supporting Th1 polarization in chronic ITP, recent reports show that Th17 cells have more potent than Th1 cells in inducing autoimmune disease. *Aim.* The present study aims to investigate the roles of Th cells cytokines involved in pediatric patients with chronic ITP. *Methods.* After approval of institutional ethic committee and written inform consent obtained from patients and/or patients' parents, 57 pediatric patients with chronic ITP and 28 healthy controls were enrolled. Patients was divided into three groups based on their platelet counts at the time of the study: (i) 'active disease' $<50\times10^9$/L (n=23), (ii) 'stable disease' $50\text{-}150\times10^9$/L (n=23), (iii) 'in remission' $>150\times10^9$/L (n=11). Plasma concentration of Th1 (IFN-γ, IL-2), Th2 (IL-4, IL-10), Th3 (TGF-β1), and Th17 (IL-17) cytokines were investigated by enzyme-linked immunosorbent assay. *Results.* IFN-γ were significantly increased in patients with active ($P < 0.001$) and stable disease ($P < 0.026$) when compared to controls. Otherwise, there were no significant differences of IL-2, IL-4 and IL-10 between patients and controls. IL-17 was significantly increased in patient groups ($P = 0.011$). In addition, there was a positive correlation of IL-17 and IFN-γ levels in ITP patients ($r= 0.640$, $P<0.001$). Reduced TGF-β1 expression was observed in patients with active ($P < 0.001$) and stable disease ($P = 0.001$), and its level was positively correlative to the platelet count ($r= 0.355$, $P=0.007$). *Summary/Conclusions.* In addition to Th1 polarization, the study shows correlative elevation of IL-17 and IFN-γ could be an important dysregulation of cellular immunity in pediatric patients with chronic ITP. Moreover, Th3 cytokine (TGF-β1) is considered as a bystander immune suppression associated with remission of the disorder.

0999

SUCCESSFUL MANAGEMENT OF GESTATION AND LABOR IN A 33-YEAR-OLD WOMAN WITH BERNARD SOULLIER SYNDROME

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Background. Bernard Soullier Syndrome (BSS) is a rare qualitative and quantitative autosomal recessive platelet disorder. It is usually associated with prolonged platelet functional tests and low platelet count with giant sized platelets. In BSS GPIb-IX-V membrane glycoprotein is absent or decreased and the result is deficient binding of vWF to the platelet membrane at sites of vascular injury. Our patient presented hemorrhagic symptoms since she was 18 months old with an average platelet count of 40×10^3/μL and an increased platelet volume that required multiple platelet transfusions throughout her childhood. On December 2007 she consulted for pregnancy. Platelet count was 40×10^3/μL, antiplatelet antibody studies were negative for membrane glycoprotein but positive for antiHLA-1 antibodies against 98% of tested donors. Following these tests HLA compatible donors were sought. Cesarean section was performed on the 38th week in order to avoid fetal hemorrhagic complications. On the day of surgery two platelet aphaeresis were transfused one hour before the procedure and one every 12 hours after. The next 4 days one apheresis was transfused every 48 hours. The fetus was born asymptomatic with a normal platelet count and volume. *Aim.* We present a case of successful management of gestation and labor in a 33-year-old woman with BSS. *Methods.* Ristocetin induced platelet agglutination test and membrane glycoprotein study were performed. *Results.* Lysed platelets were incubated with anti-GPIbα SZ2 monoclonal antibody. Platelets were subsequently incubated with anti-αIIb and anti-β3 to verify that the patient and control had the same amount of protein. Flow cytometry analysis revealed that patient αIIb and β3 were correctly expressed and GPIb_ remained undetectable. Control and patient DNA segments were amplified by PCR to obtain GPIbα sequence. Sense segment revealed simultaneous presence of G-C nucleotides at position 688 and T-A nucleotides at position 715. These mutations translate into a heterozygotic Ala200Pro and Cys209Ser replacement in the mature peptide. Anti-sense segment analysis confirmed the presence of these two mutations. Ala200Pro mutation has not been previously reported in BSS. Heterozygotic Cys209Ser mutation has not been described alone either. *Conclusions.* We present a case of successful management of gestation and labor in a young woman with BSS. Flow cytometry analysis reveals absence of GPIα and under expression of GP IX. Molecular analysis shows 2 mutations, one of them; Ala200Pro had not been previously reported. Up to date there are only 12 published cases of labor related BSS. Most of them presented severe hemorrhagic complications. None was molecularly diagnosed. Platelet transfusion is the main treatment in BSS. Antiplatelet antibody study should be performed as they are frequently increased in these patients. Management of this condition requires a multidisciplinary approach involving coordination between gynecology and hematology departments. BSS is a rare condition (less than 1 case per million) and there are currently no well-established protocols for the management of BSS in pregnancy.

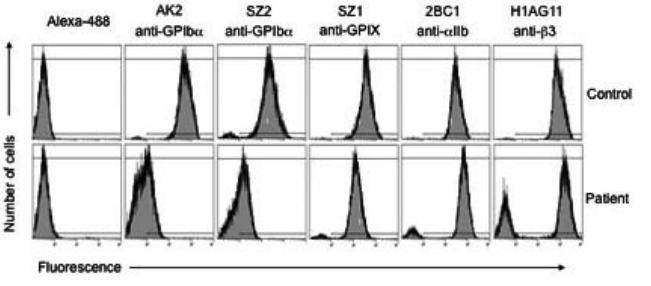

Figure 1. Flow cytometry.

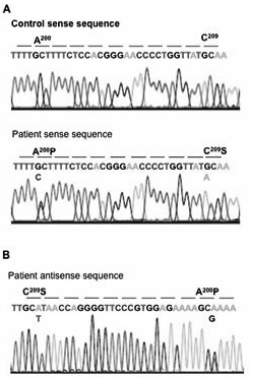

Figure 2. Gplba gene mutation.

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ASSOCIATION OF PLATELET PARAMETERS WITH GLYCOSYLATED HEMOGLOBIN IN DIABETIC PATIENTS WITH MYELODYSPLASIA AND NORMAL PLATELET COUNT

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Background/Aim. Altered thrombocyte morphology and function have been reported in patients with diabetes mellitus type 2. High mean platelet volume (MPV) values are related with an increased risk of devel-

oping micro- and macrovascular diabetic complications such as cardiovascular disease. The aim of the present study was to determine the associations between platelet markers and glycemic indices such as glycohemoglobin (HbA1c) and fasting glucose, hypertension and coronary heart disease (CHD) in diabetic patients with primary myelodysplastic syndrome (MDS), in patients with diabetes mellitus type 2 and in controls. *Methods.* This cross-sectional study included thirty incident, histologically confirmed cases with primary MDS with normal platelet count (>150×10^9/L) and non-insulin dependent diabetes (Group A), thirty non-insulin dependent diabetic patients (Group B) and thirty non-diabetic healthy controls (Group C) without any neoplastic and infectious conditions matched on age (±5 years), gender and time of diagnosis to Group A and B. Platelet count, MPV and platelet distribution width (PDW) were measured in blood samples anticoagulated with sodium citrate within 90 min after collection by venipuncture. Each measurement was performed in two different automated blood cell counters (Sysmex XE 2100 and Cell Dyn 1700). HbA1c was measured using high-performance liquid chromatography (G7 TOSOH Glycohemoglobin analyzer, TOSOH Corporation, Japan). Fasting blood glucose was determined by glucose oxidase methodology. Statistical analysis of the data was performed using SPSS for Windows version 10 statistical software package. *Results.* MPV and PDW in diabetic patients were significantly higher than those in MDS diabetic patients (P<0.001) and controls (P<0.001). In the group of MDS patients with diabetes (Group A), there were no correlations between MPV and fasting glucose (r=-0.157, P=0.408), and MPV and HbA1c (r=0.014, P=0.941). MPV and PDW were statistically significantly higher in MDS patients with hypertension (P=0.03 and 0.02 respectively) and in MDS patients with CHD (P=0.02 and 0.05 respectively). Adjusting for age, gender, body mass index (BMI), platelet number, CHD and hypertension, HbA1c as well as fasting blood glucose did not present any statistically significant association with MPV (P=0.55 and P=0.26 respectively) and PDW (P=0.66 and P=0.28 respectively) in MDS diabetic patients. Adjusting for the aforementioned confounding factors, HbA1c and fasting glucose were significant predictors of MPV and PDW in diabetic patients (P<0.001 and P=0.014, respectively). In controls, glucose, platelet count and hypertension predicted significantly platelet morphology. *Conclusions.* MPV and PDW are associated with glycemic control indices in diabetic patients reflecting association with macrovascular disease. HbA1c and glucose were not correlated with MPV or PDW in diabetic MDS patients suggesting that other factors inherent to bone marrow dysplasia are involved in platelet morphology.

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EVALUATION OF ACTIVATION AND APOPTOSIS PHOTOCHEMICAL TREATED AND GAMMA-IRRADIATED PLATELET CONCENTRATES

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Background. The safety of blood components is the main task of transfusion medicine. Photochemical treatment (PCT) of apheresis platelet concentrates (A-PLT) for inactivation of pathogens and residual leukocytes with amotosalen and long-wavelength ultraviolet A (UVA) light has been developed to prevent transfusion complication, associated with pathogens and donors leukocytes. Gamma irradiation (GI) of blood components, including A-PLT, is used for inactivation of allogenic T-lymphocytes. The accepted dose of GI in Russia is 25 Gy. *Aim.* The aim of this study is to evaluate and compare the functionality and viability of PCTed platelets (PLTs) and GIed PLTs on the base of apoptosis and activation markers. *Material and methods.* 12 samples of A-PLT were investigated before and after PCT, 10 samples of A-PLT were investigated before and after GI . PLTs for PCT were prepared in 35% plasma and 65% platelet additive solution, pre-PCT yield was 2.5-6.0×10^{11} in volume 320±5 Ll. Each unit was treated with 150 μM amotosalen and 3,6 J/CM^2 UVA light followed by 4-5 hours of incubation with a compound adsorption device. A-PLT concentrates for GI were prepared in the same condition and were irradiated with 25 Gy during the first 24 hours after collection. All samples were investigated by flow cytometry. PLT apoptosis was measured by phosphatidylserine (PS) exposure with FITC-labeled Annexin V. PLT activation was measured by surface P-selectin (CD62P) expression. *Results.* The pre-treatment level of CD62P+ PLT wa.0±1.8%. P-selectin expression was decreased to19.3±2.8% after PCT. The initial level CD62P$^+$ PLT was maintained unchanged after 25 Gray of gamma irradiation. Pre-treatment and post-treatment levels of P-selectin (CD62P) expression were compatible with viable PLT function. PS exposure at the membrane surface was detected on 8.8±2.0% PLTs and was not changed significantly after PCT (10.7±2.6%). Increasing of Annexin V binding was detected only in some GIed samples and was <2.5-fold, but it was not exceed the initial maximum level. *Conclusions.* PCT and GI (25 Gy) of A-PLT was not lead to increasing of PLT apoptosis and activation, like markers of viability and functionality. PCTed and GIed A-PLT concenrates were comparable with untreated controls.

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PERIPHERAL BLOOD PHENOTYPIC ANALYSIS IN AUTOIMMUNE THROMBOCYTOPENIC PURPURA

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Background. Autoimmune Thrombocytopenic Purpura (AITP) is a common acquired hematologic disorder. Accumulating evidence suggests that different T-cell dominant and B-cell dominant pathophysiologic mechanisms may be present and responsible for the chronic, refractory form of the disease. *Aim.* In this study we examined peripheral blood phenotypic analysis abnormalities as well as possible correlations among circulating lymphocyte subtypes, age, sex, serological findings and course elements of AITP. *Methods.* Twenty adult AITP patients (15 women and 5 men, median age 32.5 years) and three children (2 boys and 1 girl, median age 14 years) were studied retrospectively. Peripheral blood phenotypic analysis data were compared to those of 20 age- and sex-matched healthy volunteers. All tests had been conducted either at diagnosis or during relapse after a minimum 3 months drug therapy free period in our department's laboratories. Data were statistically processed using appropriate methods and software. *Results.* Although no differences were observed between well-responding and relapsing patients, a significant negative correlation between CD20$^+$/CD23$^+$ B-cell levels and the number of the patients' relapses per year, as well as a significant positive correlation between high CD19$^+$ and CD22$^+$ levels and the frequency of splenectomy were noted. CD5$^+$ and CD7$^+$ T-cell levels were significantly reversely correlated with the frequency of positive ANA and IgG anti-Cardiolipin autoantibodies. Patients beyond 60 years of age had significantly lower levels of CD2$^+$ and CD3$^+$ T-cells as well as significantly higher levels of the CD5$^+$/CD19$^+$ co-expression. Finally, CD19$^+$, CD20$^+$, CD22$^+$ B-cell levels, CD19$^+$/CD79b$^+$, CD19$^+$/CD25$^+$ and CD20$^+$/CD23$^+$ markers and the Fmc7$^+$/CD11c$^+$ co-expression were significantly elevated among patients in comparison to healthy subjects. No differences between the two groups were observed as far as NK cells and T suppressor cells are concerned. *Conclusion.* Our findings suggest that B-lymphocyte abnormalities as well as T-cell defects related with age may be responsible for the pathogenesis and outcome of the disease, although additional studies will be useful in order to elucidate the biology of AITP.

1003

DETERMINATION OF REGULATORY CELLS IN PATIENTS WITH IDIOPATHIC THROMBOCYTOPENIC PURPURA USING FLOW CYTOMETRY

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Background. ITP (Idiopathic Thrombocytopenic Purpura) is an autoimmune disease with multi-dysfunctional pathophysiology. Although, production of autoantibodies by autoreactive B cells is considered as the primary immunologic defect, there are several other mechanisms which also seem to play critical role. Such a mechanism are regulatory T cells which participate in peripheral tolerance and seem to interfere in autoimmune diseases. *Aims.* Our primary aim was to detect and measure the percentage and absolute numbers of naturally arising CD4$^+$CD25^{high}FoxP3$^+$ T regulatory cells in peripheral blood of patients with ITP and healthy donors using flow cytometry. We also investigated the subpopulations of CD4$^+$CD25^{high}FoxP3$^-$ and CD4$^+$CD25$^-$FoxP3$^+$ cells. *Methods.* In order to identify the subpopulations of T regulatory cells, we used two different protocols. The first one concerned the determination of transcriptional factor FoxP3 in the subpopulations of CD4$^+$CD25^{high} and CD4$^+$CD25$^-$ cells. Additionally, as a confirmation we developed a second protocol to determine the interleukin-7 receptor (CD127). *Results.* Comparing the results of 21 patients with ITP with

those of 20 healthy donors, the absolute number of naturally arising T regulatory cells $CD4^+CD25^{high}FoxP3^+$ as well as that of $CD4^+CD25^{high}FoxP3^-$ and $CD4^+CD25^-FoxP3^+$ appeared significantly decreased in patients with ITP who were either in remission or in active disease. *Summary/Conclusions.* Our findings showed that patients who suffer from ITP have an impairement concerning not only thymus derived $CD4^+CD25^{high}FoxP3^+$ T regulatory cells, but also $CD4^+CD25^{high}FoxP3^-$ and $CD4^+CD25^-FoxP3^+$ subpopulations.

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HEPARIN-INDUCED PLATELET THROMBOCYTOPENIA: THE ROLE OF PLATELETS GENETIC POLYMORPHISMS

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Background. Heparin induced thrombocytopenia (HIT) is a severe complication of heparin therapy, characterized by great reduction of platelet's count, that may be complicated in 30-50% of cases by thrombosis (HITT), either arterial or venous. Heparin treatment can induce antibodies (Ab, IgG class), that recognize a complex of heparin (H) and PF4. H/PF4/Abs bind platelets receptor FcgRIIA, inducing platelet activation and aggregation, by modulating the affinity of GpIIb-IIIa for fibrinogen. PECAM-1 has been shown to negatively regulate activation downstream FcγRIIA, but the mechanism is still unclear. Moreover the receptor FcγRIIIA, expressed on macrophages, seems to play an important role on the clearance of IgG-coated platelets. However HIT and HITT does not develop in all patients, the onset of clinical syndrome depends on different factors: heparin type, antibodies functionality, other autoimmune pathologies and individual genetic variations. We studied four different polymorphisms, located in the receptors previously described: FcgRIIA-H131R, GpIIb/IIIa-HPA1, PECAM1-L125V (in linkage-disequilibrium with S563N and R670G) and FcγRIIIA-F158V. *Aims.* The aim of the present study is to understand if polymorphisms of platelets receptors may influence the clinical features of patients who develop H/PF4/Abs, HIT or HITT, combining immunological, functional and genetic studies. *Methods and patients.* First we used ELISA to determine the presence of H/PF4/Ab in plasma samples, than we used heparin induced platelet activation (HIPA) as functional test to understand whether the antibodies found were able to activate donor's platelets. Using the 4T score for HIT and the result of immunological and functional tests, we define three groups: 51 H/PF4/Ab patients: antibodies not able to activate platelets-no thrombocytopenia. 50 HIT patients: Abs able to activate platelets + thrombocytopenia. 53 HITT patients: Abs able to activate platelets + thrombocytopenia + thrombosis. We used molecular biology techniques to determine the genotype of polymorphisms: allele-specific-PCR for FcγRIIA-H131R, PECAM1-L125V and FcγRIIIA-F158V; an allelic discrimination real-time-PCR using taqman probes for HPA1. Hardy-Weinberg equilibrium was tested for each polymorphism. Allele or genotype frequencies between patients were compared by the χ^2 test; we use Multiple Regression Analysis for confront between polymorphisms. *Results.* Comparing the polymorphisms frequencies between the patients groups (H/PF4/Ab; HIT; HITT) we found statistical differences between HIT and HITT. R/R131 genotype frequency (FcγRIIA) is increased in HITT ($P<0,05$), the same for A/B genotype frequency (GpIIIa-HPA1) in the same group ($P<0,05$). The frequency of the polymorphic setting VNG (V/V125-N/N563-G/G670) for PECAM1 is also increased in HITT group compared with the other groups, but p-value is not statistically significative. There were no genotype differences comparing HIT with H/PF4/Ab group. *Conclusions.* We suppose that platelets R/R (receptor FcγRIIA), cleared less efficiently than H/H ones, can circulate longer enhancing the risk for HIT thrombosis; and that the setting VNG for PECAM1 can have less inhibitory activity on FcγRIIA. Furthermore theallele for HPA1 is a known risk factor for thrombosis. We found that R/R131 associated with HPA1 have a relation with HITT but with a p-value (0,07) near significance. We think that increasing our cases we could obtain a significant association between our polymorphisms and HITT.

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EVALUATION OF PLATELET FUNCTION UNDER HIGH SHEAR CONDITIONS IN IMMUNE THROMBOCYTOPENIA DURING ELTROMBOPAG TREATMENT

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Introduction. Eltrombopag, a small, non-peptide thrombopoietin receptor agonist, has been proven to raise platelet counts and diminish bleedings in immune thrombocytopenia (ITP). Data on its effect on platelet function are scarce. *Objectives.* We examined functional characteristics of Eltrombopag-induced platelets in 3 patients enrolled in the RAISE Study. *Methods.* Platelet function was assessed before treatment and in weekly intervals during 12 weeks of treatment. Platelet adhesion under high shear conditions was evaluated by IMPACT-R *ex vivo* as well as after *in vitro* ADP and TRAP-6 induced activation. P-selectin expression was measured without and after activation with suboptimal concentrations of TRAP-6 and ADP. *Results.* Expression of P-selectin [%] *ex vivo* was elevated (20.95, 27.01, 29.90) before treatment and declined to normal (11.41, 11.45, 5.85) after 5 weeks of treatment at platelet counts [G/l] of 161, 65 and 230, respectively. TRAP-6 induced platelet activation normalised (reaching >80%) within the first week of treatment in two cases and stayed in subnormal range in one. P-selectin expression upon activation with ADP as well as the agonists´ induced *in vitro* activation under high shear conditions showed no interrelation with rising platelet counts. *ex vivo* platelet adhesion under high shear conditions [%] was very low before Eltrombopag treatment (0.9, 0.7 and 1.6) and increased to normal (>4%) in all patients as platelet counts rose to > 100 G/l. *Conclusion.* Compared to baseline our results show decreasing values in P-selectin expression corresponding to reduced platelet activation *in vivo* and an increase of platelet adhesion under high shear conditions after Eltrombopag-induced platelet increase.

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SINGLE NUCLEOTIDE POLYMORPHISMS OF THE INFLAMATORY CYTOKINE GENES INTERLEUKIN-1?, TUMOR NECROSIS FACTORS ? AND ? IN ADULT PATIENTS WITH IMMUNE THROMBOCYTOPENIC PURPURA

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Background. Immune thrombocytopenic purpura (ITP) is an autoimmune disease characterized by thrombocytopenia due to platelet autoantibodies specific for platelet membrane glycoproteins, such as GPIIb/IIIa, GPIb/IX and GPIa/IIa. These autoantibodies cause an accelerated clearance of opsonized platelets by phagocytes in the reticuloendothelial system. The etiology of ITP remains unclear, but both genetic and environmental factors are thought to play role in the development of the disease. Several genes involved in immune system regulation like cytokine genes, Fc gamma receptor genes and HLA genes, as well as some infective agents like hepatitis C virus, HIV virus, and helicobacter pylori have been associated with susceptibility to ITP in several studies. *Aims.* The aim of our study was to investigate a possible association of some single nucleotide polymorphisms (SNP) in genes for interleukin beta (IL-1β -511 C/T), tumor necrosis factor beta (TNF β +252 G/A) and tumor necrosis factor alpha (TNF ασ -308 G/A) with ITP. *Methods.* We have analyzed 35 adult patients with ITP (7 men and 28 women) with average age of 48,8±17,8 and 100 healthy matched controls. Informed consent was obtained from all participants. The median follow up of the patients was 8.6 years. DNA was isolated from peripheral blood mononuclear cells with standard phenol-chloroform extraction. Genotyping was performed by using PCR and RFLP methods. *Results.* Our results demonstrated significantly different distribution of the TNF β genotypes in patients with ITP (n=35; G/G=0, A/G=9, A/A=26) comparing with controls (n=100; G/G=14, A/G=29, A/A=57), P=0.043. Allele frequencies for TNF β (+252 G/A) were also significantly different in patients with ITP (A allele 87,2%, G allele 12.8%) comparing with controls (A allele 71,5%, G allele 28,5 %), P=0.014 with Yates correction. We didn't found significant differences in the genotype distribution or allele frequencies for two other genes. Allele frequencies for TNF α (-308 G/A) were 5.7% for A allele and 94.3% for G allele in patients and 12% for A allele and 88% for G allele in controls, P=0.208 with Yates correction. For IL-β (-511 C/T) allele frequencies were 65.7% for C allele and 34.3% for T allele in patients and 69.5% for C allele and

30,5% for T allele in controls, P=0.662 with Yates correction. *Conclusion.* The obtained data indicate that the A allele of TNF β+252 is more frequent in patients than in controls and that this polymorphism may play role in disease susceptibility. Our results are similar with previously reported results in Caucasian patients with childhood chronic ITP by Foster *et al*. (2001), but different from the results reported by Satoh *et al*. (2004) in Japanese patients with ITP. The reason for this difference is unclear. Possible explanation may be the differences in patient's ethnic background. These results also implicate that multiple other factors play role in the etiopathogenesis of immune thrombocytopenia.

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SERUM PF4 AND HOMOCYSTEINE LEVELS IN CONTEXT OF PATHOGENETIC AND PROTHROMBOTIC MUTATIONS IN ET PATIENTS.

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Background. Platelets of ET(Essential thrombocythemia) patients are characterized by specific morphological, biochemical and metabolic defects. The most frequent dysfunction is a complete loss of platelet responsiveness to epinephrine. On the other hand there are some evidence of *in vivo* platelet activation. Active platelets secrete platelet factor 4 which is the most important marker of their activation. *Aims.* In this study we assessed serum PF4 and homocysteine levels in context of presence of pathogenetic and prothrombotic mutations , bleeding and thrombotic complications and correlation between these parameters and main coagulation factors activity. *Methods*. We have examined 32 patients with ET(24 females and 8 males , mean age 56.0±14.2). The control group(CG) consisted of 20 healthy person: 6 males and 14 females (mean age 41.4±8.3). In 11 patients from ET group 19 thrombotic complications occurred and in 7 ET patients bleeding episodes were noticed. We evaluated PF4, homocysteine, folic acid, witamin B12, cholesterol and triglicerydes serum levels. We searched for JAK2 V617F, Factor V Leiden, prothrombin gene and MTHFR gene mutations. We assessed plasma activity of fibrinogen, factors: VIII, XII, vW; AT , protein C and S. Platelet aggregation was measured using ADP and epinephrine as agonists. *Results.* Concentration of PF4 was higher in patient serum as compared to the CG (median 24500, P25-75%: 22750-25500 *vs.* 17500,12250-22650 Iu/mL, P<0.001). To eliminate the influence of elevated PLT amount on PF4 concentration a ratio of PF4 per million PLT was calculated. The ratio PF4/PLT was significantly lower in ET group as compared to CG(median 30.4; P25-75%: 27.8-38.9 IU/106PLT *vs.* 62.9; 45.1-94.6; P=0.000002). The PF4/PLT ratio was in positive correlation with vWF Ag (P<0.05) and in negative correlation with triglycerides concentration (P<0.05). Median homocysteine serum level was similar in both group (ET 9.77 umol/l,P25-75% 8.11-12.4 *vs.* CG 9.21umol/L, P25-75%7.94-10.60 umol/L). In 31% of patients of ET group hiperhomocysteinemia was detected, only in 2 person simultaneously with MTHFR gene mutation. One of ET patient, a 76 year- old woman underwent three times heart infarct. Other risk factors were: hypertension , JAK 2 mutation, extremely high homocysteine level (24.6 umol/L) without MTHFR gene mutation. The coronarography revealed normal coronary artieries. The second patient, a 79 year-old man, with very high homocysteine level (21.7umol/l) had obliterative atheromatosis diagnosed at the some time as ET. No JAK2 and MTHFR gene mutations was detected. Erytromelalgia was complained by 65% of ET patients and correlated with higher level of witamin B12 and cholesterol. 57% of ET patients and 7% person from CG displayed a complete loss of platelet responsiveness to epinephrine. *Conclusion.* Platelets of ET patients release significantly less PF4 during thrombus formation than platelets of control group, which may indicate impaired platelet function. A very high homocysteine level in some ET patients may contribute to thrombotic complications especially in connection with other risk factors.

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IDENTIFICATION OF PATIENTS WITH THROMBOCYTOPENIA IN PADUA UNIVERSITY AND CITY HOSPITAL THROUGH THE ADMINISTRATIVE DATABASE

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Background. The use of administrative database can be a powerful method to estimate the disease prevalence in a population. We identify patients with thrombocytopenia having an International Classification of Disease, 9th Revision, Clinical Modification (ICD9CM) code of 287.3 (immune thrombocytopenic purpura), 287.4 (secondary thrombocytopenia) and 287.5 (unspecified thrombocytopenia). *Methods.* The administrative database, we used, contains information on all patient admission and discharge including patient demographics, diagnostic and procedure codes, functional areas of discharge and the diagnoses related group code. We evaluate patients discharged from the Padua University-City Hospital in Padua from 2004 to 2007 to identify all cases of thrombocytopenia as a primary or secondary diagnosis, the diseases associated to primary or secondary diagnosis, therapeutics procedures and bleeding complications. Results. In 301 patients (160 female, 53%, 141 male, 47%) have been used one of the ICD9CM related to thrombocytopenia: 199 as primary diagnosis and 102 as secondary one; 154 patients were billed with 287.3 (131 as primary diagnosis, 23 as secondary), 79 patients with 287.4 (40 as primary and 39 as secondary one) and 68 were billed with 287.5 (28 as primary diagnosis, 40 as a secondary diagnosis). 84 patients with immune thrombocytopenia (ICD-9-CM code 287.3) were discharged from internal medicine units, 40 from pediatric area, 15 from haematology and oncology area, 5 from medical specialties, 7 from general surgery, 2 patients from cardiovascular area and 1 from clinical surgery. 51 patients with secondary thrombocytopenia (ICD-9-CM code 287.4) were discharged from internal medicine units, 18 from haematology and oncology area, 4 from pediatric area, 3 from medical specialties, 1 from cardiovascular area, 1 from clinical surgery and 1 from general surgery. 27 patients with unspecified thrombocytopenia (ICD-9-CM code 287.5) were discharged from internal medicine units, 18 from haematology and oncology area, 11 patients from pediatric area, 6 from medical specialties, 5 from general sugery, 1 from clinical surgery. The analysis of associated diagnoses at discharge showed a wide heterogeneity; 32% were missing; 10% regarded cancer; 9% regarded blood and hematopoietic diseases, 12% concerned cardiovascular and respiratory system diseases, 8% regarded digestive diseases, 7% regarded infective diseases, 4% regarded endocrine diseases and 18% regarded miscellany. 27(9%) patients had bleeding complications and most were over 60 years. 54 patients (18%) were subjected to therapeutic procedures related to the thrombocytopenia: 10 of the patients underwent to splenectomy; 26 to transfusion of packed red cells or platelets; 14 were subjected to infusion of high doses immunoglobulins; 4 were subjected to therapeutics plasma-exchange. *Conclusions.* The incidence of thrombocytopenia calculated by the rate of hospitalization of the area was 3,2/100.000/year. Therefore trough the administrative database it's possible identify patients with thrombocytopenia. Most discharged patients are from medicine (internal medicine and medical specialties). Bleeding complications are not so high if related to the large number of therapeutic interventions and it may be assumed that there is no registration of bleeding events. It would be interesting to compare data with medical records to assess the accuracy and the truthfulness of the discharge schedule.

Red cell and iron 2

1009

INCREASED EFFLUX OF OXIDIZED GLUTATHIONE (GSSG) CAUSES GLUTATHIONE DEPLETION AND INCREASED SUSCEPTIBILITY TO OXIDATIVE STRESS IN SICKLE ERYTHROCYTES

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Introduction. Sickle cell disease (SCD) is characterized by increased oxidative stress, playing an important role in the pathophysiology of acute and chronic organ complications in sickle cell patients. Sickle erythrocytes are both an important source and target of reactive oxygen species (ROS). Levels of both total and reduced form of glutathione (GSH), a major antioxidant, are decreased in sickle erythrocytes. The mechanism leading to glutathione depletion in sickle erythrocytes is not known yet. After reacting with ROS, GSH is oxidized into glutathione-disulfide (GSSG) and can be transported actively out of the erythrocyte. *Aims.* We questioned whether under oxidative conditions, GSSG efflux is increased in sickle erythrocytes. *Methods.* Erythrocytes of 18 homozygous sickle cell patients and 9 race-matched healthy controls were stimulated with 2,3-dimethoxy-l,4-naphthoquinone (DMNQ), which induces intracellular ROS generation, to stimulate GSH consumption. Intra- and extracellular levels of GSH and GSSG were measured at baseline and after 90, 150 and 210 minutes DMNQ stimulation. *Results.* While comparable at baseline, GSSG production and efflux (μM) were higher in sickle erythrocytes after 90 (P=0.013 and NS respectively), 150 (P<0.0001 and =0.004 respectively) and 210 minutes (P=0.002 and P<0.0001 respectively) DMNQ stimulation. In contrast to control erythrocytes, where GSH levels remained unchanged, GSH in sickle erythrocytes decreased significantly during DMNQ stimulation. Adding multidrug resistance-associated protein-1 (MRP-1) inhibitor (MK571) to erythrocytes blocked GSSG efflux in both sickle and normal erythrocytes. *Conclusions.* GSSG efflux, mediated by MRP-1, is increased in sickle erythrocytes, resulting in net loss of intracellular glutathione and thus higher susceptibility to oxidative stress. The increased GSH consumption upon oxidative exacerbation in sickle erythrocytes suggests a reduced antioxidative reserve capacity in SCD.

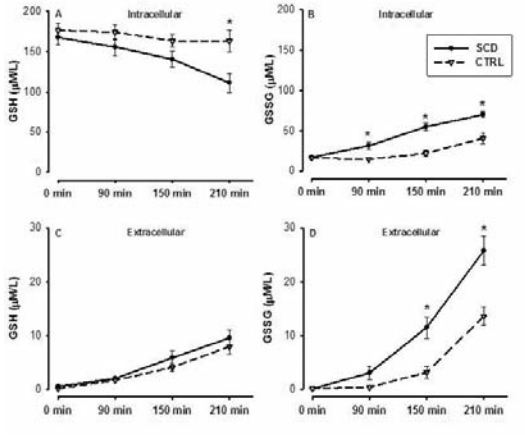

Figure. GSSG efflux upon oxidative stimulation.

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PENTRAXIN 3 AS MARKER OF DISEASE SEVERITY DURING SICKLE CELL PAINFUL CRISIS

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Background. The painful crisis accounts for the majority of sickle cell disease (SCD) related hospital admissions. The prototypic long pentraxin 3 (PTX3), an acute phase protein, is elevated in patients with inflammatory and ischemic states. *Aims.* As the sickle cell painful crisis is associated with both inflammation and tissue ischemia, we questioned whether plasma PTX3 levels are increased and associated with disease severity during painful crisis. Furthermore, since PTX3 up-regulates endothelial expression of tissue factor we studied PTX levels in relation to markers of coagulation activation. *Methods.* Plasma levels of PTX3, ultra-sensitive C-reactive protein (US-CRP), prothrombin fragment 1+2, thrombin-antithrombin (TAT) complexes and von Willebrand Factor antigen and serum levels of soluble vascular adhesion molecule-1 were determined in 105 asymptomatic sickle cell patients, 33 patients during painful crisis and 30 race matched healthy controls. *Results.* Plasma PTX3 levels (ng/mL) were comparable between patients in asymptomatic state and healthy controls, but significantly higher during painful crisis (P<0.01). Also in paired sample analysis of 24 patients, PTX3 levels increased during painful crisis (P<0.001). US-CRP levels were higher in asymptomatic patients compared to controls (P<0.0001) and increased further during painful crisis (P<0.0001). PTX3 levels at presentation with painful crisis correlated significantly to the duration of related hospital stay (rs=0.43; P=0.013), whereas US-CRP levels did not. The hemolytic rate, but not PTX3 levels, was related to markers of hypercoagulable state in sickle cell patients. *Conclusion.* The significant increase of plasma PTX3 levels during sickle cell painful crisis and their correlation to the duration of subsequent hospital stay suggest that PTX3 is a potential marker of painful crisis severity. PTX3 seems to have no significant role in coagulation activation in SCD.

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INTRONIC LINE-1 INSERTION IN THE BETA-GLOBIN GENE CAUSES BETA-THALASSEMIA DUE TO ABERRANT SPLICING, NONSENSE-MEDIATED DECAY AND DECREASED RATE OF BETA-GLOBIN L1 ALLELE TRANSCRIPTION

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Background. β-thalassemia is a common hereditary hemoglobin disorder characterized by quantitative reduction of functional β globin chains. The patients, mother and daughter of Ukrainian descent exhibited typical laboratory features of β-thalassemia trait. Molecular analyses revealed that the full-length (6 kb) retrotransposon L1 was inserted in the antisense orientation into the intron-2 of the *β globin* gene (collaboration with Dr. J. Prchal, Salt Lake City, UT). The total level of expression of the affected *β globin* gene transcript was reduced to 10-15% of the total *β globin* mRNA and thus leading to β$^+$-thalassemia. Based on recently published data we hypothesize that RNA production of mutated gene was affected by the combination of several events. Aim. The exact mechanism by which the intronic insertion of transposable element attenuates human gene expression is not known. We have tested several hypotheses which could contribute to the final elucidation of the molecular mechanism leading to the thalassemia phenotype caused by mutated *β globin* gene. *Methods.* Interspecific hybrids of the propositus´ lymphocytes and mouse erythroleukemia (MEL) cells were generated in order to evaluate expression of the mutant and unaffected *β globin* gene allelles (laboratory of Dr. T. Papayannopoulou, Seatlle, WA). The total RNA from differently treated (emetine, 5-aza-2'-deoxycytidine (Sigma)) *in vitro* cultured cell hybrids were isolated and *β globin* transcripts were analyzed by Real-Time qPCR with gene-specific primers. Nuclei from hybrid MEL cells were isolated to performed nuclear run-on assay. Bisulfite modification was done on genomic DNA from MEL hybrids and the enhancer region of *β globin* gene was amplified, PCR products were subcloned and sequenced. *Results.* We demonstrated that the observed reduction in steady-state level of *β globin* mRNA is partially caused by aberrant splicing followed by activation of nonsense-mediated decay (NMD) pathway, leading to increased degradation of aberrant *β globin* mRNA variants. Reduction in expression of *β globin* mRNA from *β globinL1* allele comes also from altered rate of transcription. We performed PCR-based nuclear run-on assay and forty minutes of *in vitro* transcription revealed 30% decrease in *β globinL1* allele transcription rate compared to wild-type *β globin* allele. We also observed the *β globinL1* 3' enhancer sequence was fully methylated. However, treatment with a demethylating agent did not increase the expression of the *β globin* transcript of the *β globinL1* gene. Therefore the methylation of the *β globinL1* 3' enhancer sequence is only a secondary event probably associated with enhancer displacement by L1 insertion. The other known mechanisms of intronic L1-mediated gene disruption

as premature polyadenylation and gene breaking were not detected in our case. *Conclusions.* The molecular mechanisms of β+-thalassemia phenotype caused by intronic insertion of LINE-1 in the *β globin* gene seem to be a combination of aberrant splicing, NMD and decreased rate of *β globinLA* allele transcription. We submit that retrotransposition of active mobile elements may alter gene expression and should be considered an independent molecular mechanism leading to human diseaseGrant support: NS10281-3/2009 Ministry of Health, Czech Republic.

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HEREDITARY SPHEROCYTOSIS (HS): RELATIONSHIP BETWEEN CLINICAL OUTCOME, DIAGNOSTIC TESTS RESULTS AND MEMBRANE PROTEIN DEFICIENCY. A PROSPECTIVE STUDY FROM ARGENTINA

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Background. HS may be difficult to diagnose because no positive family history is evident in around 25% of patients and accuracy of diagnostic tests is variable No predictive factor for HS outcome based on laboratory tests results is clearly established. Previous large trials simultaneously performing osmotic fragility (OF), autohemolysis (AH), hypertonic cryohemolysis (CH), eosin-5'-maleimide flow cytometry (EMA-FC), and SDS-PAGE analysis of membrane proteins have not been reported. Prevalence of each protein deficiency in Argentine patients is unknown. *Aims.* a) Assessing relationship between membrane protein defects, laboratory features and clinical outcome; b) Evaluating usefulness of CH and EMA-FC; c) Determining prevalence of protein deficiencies. No previous report concerning these subjects has been published in Argentina. *Methods.* 106 individuals (60 patients and 46 asymptomatic relatives), aged 1m-77y, belonging to 46 families were prospectively studied; 9 patients had been previously splenectomized; 42 cases were <18y; 255 healthy blood donors served as controls. Following tests were performed: OF, AH, CH, EMA-FC, and SDS-PAGE. Blood samples were collected simultaneously with samples from 6 normal controls and processed within 48 hours. EMA-FC results were expressed as percentage of decrease of mean channel fluorescence (MCF) and as increase of the coefficient of variation (CV). Diagnostic criteria for HS were evidence of hemolytic anemia plus two positive tests. In non-splenectomized patients, anemia was defined as severe, moderate or mild (hemoglobin <8, 8-10 or >10 g/dL, respectively). Relatives showing any membrane protein deficiency but otherwise asymptomatic with negative diagnostic tests were considered as HS silent carriers. *Results.* HS was diagnosed in 47 patients and 7 relatives; 16 carriers were detected. Inheritance pattern was dominant in 31, non-dominant in 14 and undetermined in 9 cases. Anemia was severe in 9, moderate in 13, and mild in 23 non-splenectomized patients. Percentages of positive tests were: EMA-FC 81.5%, CH 81.1%, OF 76.1%, SDS-PAGE 72.7%, and AH 65.9%. Only 19 patients (35.2%) were positive for all the tests. Regarding only CH and EMA-FC, at least one of them was positive in 92.6% of patients. SDS-PAGE showed single or combined protein deficiencies in 48 cases belonging to 27 families. Most frequently found deficiencies were spectrin in analysis by individuals (28.3%) and ankyrin in analysis by families (31.7%). All spectrin-deficient cases showed dominant inheritance. No significant relationship between tests results, inheritance pattern, neonatal jaundice or deficient protein was observed. Analysis of tests in relation to anemia only showed significantly higher increases of CV in moderate and severe anemia than in mild anemia or splenectomized patients (p: 0.004); a trend to significantly lower values of MCF in severe anemia than in other groups was also observed. No relationship between protein deficiencies and severity of anemia was found. *Conclusions.* A) EMA-FC seems to be a reliable predictor of severity; no other relationship between tests results and outcome was observed. B) Accuracy of EMA-FC and CH for diagnosis was higher than for other tests; simultaneous reading of MCF and CV improves EMA-FC diagnostic usefulness. C) Predominant spectrin and ankyrin deficiencies agree with reports from other Latin American countries.

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GENETIC VARIABILITY OF TMPRSS6 GENE AND ITS ASSOCIATION WITH IRON DEFICIENCY ANEMIA

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Background. TMPRSS6 (Transmembrane Protease, Serine 6) has a key role in iron homeostasis, as proved by previous studies, and TMPRSS6 mutations have been shown to greatly affect iron status. Loss of function of this gene, causing elevated hepcidin levels, is associated with iron-refractory iron deficiency anemia (IRIDA). Although the pathogenic TMPRSS6 mutations that underlie IRIDA appear to be relatively rare, many common variants have been reported in several studies. The possibility that these variants in TMPRSS6 may contribute to susceptibility to iron deficiency anemia has not yet fully understood. *Aim.* 16 patients with iron deficiency anemia refractory to oral iron therapy were genotyped for TMPRSS6 gene, in order to evaluate the association of iron deficiency anemia with single mutations/polymorphisms and haplotype combinations. Fifty healthy control individuals were selected from the general population. *Methods.* Polymerase chain reaction (PCR) and direct sequencing were used to screen for genetic variations in the TMPRSS6 gene cluster. Allele and genotype frequencies were compared between cases and unrelated controls using the χ^2 test or Fisher's exact test. The strength of the association was estimated by odds ratio of risk (OR) and 95% confidence intervals (95%CI). Linkage disequilibrium (LD) calculations and haplotype association analysis were done using the Haploview package version 4.0. *Results.* Two novel single nucleotide substitutions, H448R and F5F, and two rare known non-synonymous polymorphisms, rs2235324 and rs2235979 were found in the coding region of TMPRSS6. Moreover, twenty-seven different DNA polymorphisms, eight of which are novel, were identified in patients TMPRSS6 gene. Among them, rs11704654, rs2235324 and rs4820268 were most significantly associated with iron-refractory anemia (P<0.0001). The frequency of the C allele of rs855791 and T allele of rs2235321 were significantly increased in patients compared to healthy controls (P<0.0001, OR=5.18, 95%CI 2.21<12.11 and P=0.011, OR=3.33, 95%CI 1.27<8.71, respectively) and in LD with each other (D'=1.0, r2=0.444, CI=0.8-1.0). Among the SNPs, rs2543519, rs2543607 and rs2543633 were associated with iron-refractory anemia (P<0.001, OR=6.90, 95%CI 2.49<19.07) and in LD with each other (D'=1.0, r^2=0., CI=0.73-1.0). For rs2235454, the minor *G allele frequency was higher in the patients (0.281) than in control subjects (0.000; P<0.001, OR=6.13, 95%CI 1.98<18.96). For rs2112025 the allele frequency of minor *G was 0.906 in patients and 0.600 in control subjects (P=0.0012, OR=6.44, 95%CI 1.83<22.58). Haplotypes and haplotype blocks were derived for the patient group, the control group and the combined patient-control cohort. We divided the TMPRSS6 gene region into 3 haplotype blocks, containing 6, 8 and 2 SNPs each. Four haplotypes (three in Block 1 and one in Block 2) were observed to be linked to the iron deficiency anemia (P<0.0001, P<0.001 and P=0.0020, respectively, for haplotypes in Block 1 and P=0.0033 for haplotype in Block 2). *Conclusions.* Our preliminary results suggest a possible association of genetic variants and specific haplotypes of TMPRSS6 gene with IRIDA, and deserve further investigations in large cohorts of subjects with iron deficiency disorders.

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LOW PREVALENCE OF CARDIAC SIDEROSIS IN HEAVILY IRON LOADED EGYPTIAN THALASSEMIA MAJOR PATIENTS

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Background. Myocardial siderosis in thalassemia major remains the leading cause of death in developing countries. Once heart failure develops, the outlook is usually poor with precipitous deterioration and death. Cardiovascular magnetic resonance (CMR) technology can measure cardiac iron deposition directly using the magnetic relaxation time T2*. This allows earlier diagnosis and treatment and helps to reduce mortality from this cardiac affection. *Aims.* To find out the prevalence of cardiac siderosis among our patients who are heavily iron loaded by CMR technology and its relation to liver iron concentration, serum ferritin and left ventricular ejection fraction. *Methods.* 89 β thalassemia patients (10 to 43 years, mean age of 20.78±6.36) were recruited in this study. All patients were receiving chelation therapy of subcutaneous desferrox-

amine. Evaluation of hemosiderosis was based on CMR, liver magnetic resonance R2 and serum ferritin. *Results.* T2* values ranged between 4.3 to 53.8 ms with a mean of 28.5±11.7 ms among our study group. The left ventricular ejection fraction (LVEF) as measured by CMR ranged between 55 and 78%; mean=67.7±4.7%.and liver iron concentration (LIC) ranged between 1.5 to 56 mg/g dry weight with a mean of 26.1±13.4 mg/g. Serum ferritin varied among our study group from 533 to 22360ng/mL; mean=4510±2847ng/mL with 83.2% above 2500 ng/mL. The prevalence of myocardial siderosis (T2*<20 ms) among our patients was 22/89 patients (24.7%) whose mean age was 20.9±7.5 years with a mean T2* value of 12.7±4.4ms and LVEF of 68.6 ±5.8%. LIC and serum ferritin results were 30.9±13.5mg/g and 6120±4190ng/mL respectively. There was no correlation between T2* results and the age, LVEF, LIC and serum ferritin of this group (P=0.65, P=0.085, P=0.99 and P=0.63 respectively). Those patients with severe cardiac siderosis (T2*<10ms) constituted 7/89 (7.9%) with a mean age of 18.4±4.4 years. Although these patients had a mean T2* of 7.8±1.7 ms, the LVEF value was 65.1±6.2 % and only one patient had clinical cardiac disease (T2*=4.3 ms and LVEF =55%). LIC and serum ferritin results were 29.8±17.0 mg/g and 7200±6950ng/mL respectively. In this group of severe cardiac siderosis, T2* was not correlated to age (P=0.5), LVEF (P=0.14), LIC (P=0.97) and serum ferritin (P=0.82). *Conclusion.* There was a low prevalence of myocardial siderosis in the Egyptian thalassemia patients in spite of very high serum ferritin. T2* is the best test that can identify at risk patients who can be treated with optimization of their chelation protocols. The possibility of a genetic component for the resistance to cardiac iron loading in our population should be considered.

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AUTOMATION OF HEPASCORE AS A BIOCHEMICAL INDEX OF LIVER FIBROSIS IN PATIENTS WITH THALASSEMIA: THE IMPORTANCE OF HYALURONIC ACID

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Background. Patients with transfusion depended thalassemia major often develop liver fibrosis due to liver iron overload and/or hepatitis virus C (HCV) infection. Hyaluronic acid (HA) plays a prominent role in the pathogenesis of liver fibrosis, and the elevation of serum HA concentration is due to either increased synthesis by inflammatory cells and hepatic stellate cells or impaired degradation by sinusoidal endothelial cells (SECs) and thus is proposed as a non-invasive biomarker of liver fibrosis either by itself and/or included in the HEPASCORE formula.[1] *Patients and methods.* In this study we evaluated prospectively a screening of liver fibrosis in 201 adult patients aged 19-54 years with transfusion depended thalassemia major, based on HA measurements. 46/201 patients were HCV-mRNA(+). HA was measured with a turbidimetric assay (Wako Chemicals GmbH) applied on Siemens ADVIA 1800 Clinical Chemistry Analyser. This assay contains hyaluronic acid binding protein (HABP), which binds the HA in serum forming a complex that combines with latex particles coated by anti-HABP antibody. The above insoluble aggregate increases turbidity in the solution. The HEPASCORE was computed from the results by using the model previously published: HEPASCORE =y/(1+y) with y=exp [-4.185818-(0.0249 age (years))+(0.7464 sex (M=1, F=0)+(1.0039 _2-macroglobulin (g/l))+(0.0302 HA (μg/l))+(0.0691 bilirubin (μmol/L))-(0.0012 GGT (U/L))]. *Results.* The main results of the study showed that: a) HA levels were increased in 110/201 (55%) thalassemia patients 85.0±10.3 ng/mL, ranged from 15.0-1495.0 ng/mL, compared to 20.8 ±7.4 ng/mL reference laboratory values, P<0.001, b) HA levels were significantly higher in HCV-mRNA(+) compared to HCV-mRNA(-) patients, 192.9±40.5 *vs.* 53.0±2.7 ng/mL, P<0.0001 c) no significant correlations were found between HA levels and/or HEPASCORE with ferritin and liver iron content (LIC) assessed with MRI (p>0.324 and p>0.270, respectively). *Conclusions.* In recent years, there has been considerable interest in the use of non-invasive markers of hepatic fibrosis and cirrhosis in patients with thalassemia, because liver biopsy is an invasive and expensive procedure with potential complications. Our findings indicate that hyaluronic acid measurements contribute to the assessment of liver fibrosis in patients with thalassemia and might be helpful for further evaluation of patients with liver biopsy if this is truly needed. Furthermore, these results are in accordance with the results published recently with the other non-invasive method Fibroscan (transient elastography), indicating that liver fibrosis in thalassemia is independent from liver siderosis.[2]

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T2* EVALUATION OF IRON OVERLOAD AT 3T AND COMPARISON WITH 1.5 T

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Background. To date the gradient echo multiecho T2* MRI technique is the most robust method for the sensitive, fast, and reproducible quantification of iron overload in transfusion dependent patients. The 1.5T MRI scanner is generally used in the clinical arena. However, given that 3T scanners are becoming largely widespread, there is a growing need to assess the practicality of evaluating MIO at 3T. *Aims.* The aim of this study was to establish the relationship between T2* values at 3T and 1.5T over the range of clinical interest of tissue iron concentrations. *Methods.* Twenty borosilicate test-tube phantoms containing increasing concentrations of Fe(III)Cl3 in 0.1N standard solution of HCl were scanned at 1.5T and 3T using a T2* GRE multiecho sequence. The phantoms T2* values were calculated within a circular region of interest (ROI). Then, 24 transfusion-dependent patients enrolled in the MIOT (Myocardial Iron Overload in Thalassemia) network were scanned at 1.5T and 3T. A single transverse slice through the liver was acquired. The T2* value was determined over a large ROI of standard dimension, chosen in a homogeneous area. Three parallel short-axis views of the left ventricle were obtained using T2* GRE multislice multiecho sequence. The global heart T2* value was obtained. *Results.* Figure 1 shows the relationships between the phantom T2* values (left), the patient liver (centre) and global heart T2* values (right) at 3T and 1.5T. In each graph, the line of the best fit is indicated. A similar conversion factor (about 0.6) was identified in phantom and patient data. *Conclusions.* A strongly significant linear relationship between T2* values at 1.5T and at 3T was found for both liver and phantoms data. The significant lower goodness of the fit between global heart T2* values at 1.5T and at 3T could be explained tacking into account that the 96% of the patients had a normal global heart T2* value at 1.5T (> 20 ms), so that the range of clinically relevant T2* values was not fully covered in patient data. Moreover, measurement errors were relevant at high T2* values due to technical constraints in sequence design and presence of susceptibility artefacts.

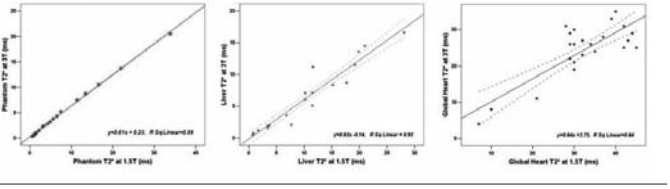

Figure 1.

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CARDIAC IRON OVERLOAD AND HEART FUNCTION BY CMR IN DIFFERENT PHENOTYPIC GROUPS OF THALASSEMIA MAJOR PATIENTS

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Background. β-thalassemia major is a genetic disorder characterized by the absence (β°) or reduced output (β⁺) of the β chains of haemoglobin. This disorder displays a great deal of phenotypic heterogeneity, not ful-

ly investigated in terms of cause-effect. *Aims.* The aim of our study was to detect if different phenotypes could be related to different levels of cardiac impairments, evaluated by cardiovascular magnetic resonance (CMR). *Methods.* We performed a retrospective review of the CMR results and of clinical data about 328 TM patients (age 29±9 years, 52% females) enrolled in the Myocardial Iron Overload in Thalassemia (MIOT) project. In the MIOT network all CMR and thalassemia centers are linked by a web-based network, configured to collect patients' anamnestic, clinical, and diagnostic data. Myocardial iron overload was assessed by using a multislice multiecho T2* approach. Cine sequences were obtained to quantify biventricular morphological and functional parameters. *Results.* Three groups of patients were identified: heterozygote (N=172), homozygote β+ (N=58), homozygote β° (N=98). No significant differences for sex and age were found among the groups. The homozygote β+ group showed higher global heart T2* values than the heterozygote and homozygote β° group (32±11 ms *vs.* 26±13 ms *vs.* 23±13 ms, P=0.001) (Figure). The homozygote β+ group showed lower LV mass indexes than the heterozygote group (54±10 g/m^2 *vs.* 60±14 g/m^2, P=0.045). The homozygote β+ group showed higher LV EF values than the heterozygote group (64±5% *vs.* 60±7 %, P<0.0001) and higher RV EF values than the heterozygote and homozygote β° group (64±5% *vs.* 59±8 % *vs.* 60±7 %, P<0.0001). *Conclusions.* The homozygote β+ TM patients showed less myocardial iron overload and a concordant better global systolic heart function and cardiac remodelling. These data support the knowledge of the different phenotypes in the clinical and instrumental management of the TM patients.

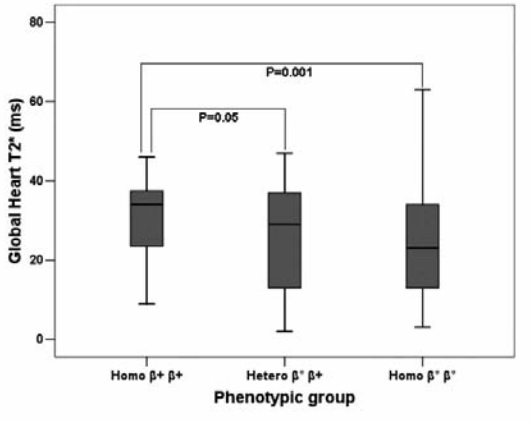

Figure.

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THE EFFICACY OF 4 DIFFERENT IRON CHELATION REGIMES IN PATIENTS WITH _-THALASSEMIA MAJOR: A 3 YEARS INSTITUTIONAL ANALYSIS

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The aim of this study was to compare the effect of different long-term chelation regimens on iron overload markers with the use of magnetic resonance imaging (MRI) and feritin in patients with transfusion-dependent beta-thalassemia major. Sixty one patients (25 men, 36 women; mean age, 28.12 +/- 8.08 years) were enrolled in the study. Four groups were formed, based on the chelation therapy received. The first group (10 patients) received deferoxamine (DFO) (30-50 mg/kg per day subcutaneously at least 5 times/week), the second group (10 patients) received deferiprone (DFP) (75 mg/kg per day orally), the third group (26 patients) received a combination of DFO (30-50 mg/kg per day, 2-3 days/week) and DFP (75 mg/kg per day, 7 days/week) and the fourth group (15 patients) received Deferasirox (30 mg/kg per day). MRI scans were acquired with an imager equipped with a 1.5 T magnet, and the data included myocardial and hepatic iron measurements obtained by means of T2*. Additionally, calculation of MRI-HIC values was based on an algorithm using liver to muscle (L/M) ratios in five axial gradient-echo sequences. All MRI scans were acguired at the beginning of the study and 3 years later. The results revealed that the Deferasirox group had significantly better Ferritin, T2* Heart, T2* Liver and MRI.HIC at the end of the study, compared with baseline (Table 1, a=P<0,05 , b=P<0,001 and c=P<0.0001). Although patients receiving combined chelation therapy did not show any statistical significant change at the end of the study as a group, a subgroup of them, consisting from 11 patients (except two treated with DFO monotherapy) with abnormal T2* heart (<20 ms) showed remarkable improvement (9.36 *vs.* 17.53 ms, P=0.04) 3 years later. *Conclusions.* There are now 3 different classes of iron chelators used mainly as monotherapy and one combination regimen with Desferrioxamine (DFO) and Deferiprone (DFP). The mode of action of each chelator is different and the choice is individualized. Combination therapy has encouraging results for patients with heart iron overload, while Deferasirox, with appropriate dosing, provides effective control of iron burden in thalassemic patients.

Table 1.

	FERRITIN (ng/ml)		T2* HEART (ms)		T2* LIVER (ms)		MRI.HIC (µmol/g dw)	
Treatment	Baseline	After 3 years	Baseline	After 3 years	Baseline	After 3 years	Baseline	After 3 years
DFO (n=10)	1118	1060	26,03	32,16	9,26	10,88	189	146
DFP (n=10)	1138	1119	39,27	39,26	6,33	9,02	222	192
COMB (n=26)	1164	1073	33,72	33,65	11,82	10,84	176,9	144,8
DFS (n=15)	1465	953,2[a]	35,46	41,5[a]	5,05	8,29[b]	212,6	118,6[c]

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SERUM HEPCIDIN LEVELS, INFLAMMATORY PARAMETERS AND IRON STATUS DURING LONG-TERM ENDURANCE TRAINING IN FEMALE RUNNERS

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Background. Exercise- induced iron deficiency is a common disorder in endurance athletes. Recently, an acute phase protein hepcidin has been suggested to have a key role in regulation of iron homeostasis. It is synthesized in liver in response to number of triggers including iron availability, inflammation, anemia and hypoxia. It was suggested that exercise induced inflammation and/or hemolysis induce hepcidin activity, which inhibits iron absorption from the gut and release from macrophages and hepatocytes. *Aims.* This study aimed to assess the influence of long term endurance training on hepcidin, inflammatory parameters and iron status in moderately trained long-distance female runners. *Methods.* Eighteen healthy women had four training session per week in training load phases. Training protocol was composed of two interval trainings (at 88-95% maximum heart rate - MHR) and two continuous runs (at 80-87% MHR). We recorded training load and measured the following parameters: Hb and RBC, serum hepcidin, interleukin (IL)-6, high sensitivity C-reactive protein (hsCRP) and iron. Samples were taken after an overnight fast at five different time-points during the eight-weeks training programme: first at Baseline Pre (BPre), than after First and Second 3 weeks Training Load (1TL, 2TL), each followed by recovery week (1R and 2R) and sixth sample was taken 10 days after the competition, Baseline Post (BPost). *Results.* During eight weeks women completed 26.3 training (3.3/week) and ran 232 kilometers (29 km/week). The time spent in heart zones was as planed (44% at 70-87% MHR, 44% at 88-100% MHR). Results are shown in mean values±SD. The levels of hepcidin decreased with time in 1TL compared to BPre (100.64±27.95 µg/L *vs.* 177.54±72.67 µg/L; P<0.001) and increased in 2TL compared to 1TL (161.09±45.67 µg/L *vs.*

100.64±27.95 μg/L; P<0.001). After the study BPost levels of hepcidin did not return to BPre (110.67±60.32 μg/L *vs.* 177.54±72.67 μg/L; P<0.001). Levels of serum iron (Fe) decreased non-significantly during 2TL (17.93±9.97 μmol /L) and 2R (16.66±8.41 μmol/L) compared to BPre (21.49±9.59 μmol/L). At the highest training points, 1TL (128.61±6.34 g/L) and 2TL (127.67±6.22 g/L), subjects had lower levels of Hb compared to BPre (133.65±7.49 g/L; P<0.001 for both). RBC levels decreased significantly at 2TL comared to BPre (4.15± 0.26 *vs.* 4.35± 0.19×10^{12}/L; P=0.01). No significant effect was found for IL-6, hsCRP at any time points. BPre IL-6 levels was below 2 ng/L and hsCRP was 1.16±1.24 mg/L. *Conclusions.* The main finding of this study is that our exercise protocol did not have significant long-term effect on inflammatory parameters as IL-6 and hsCRP. The decrease of hepcidin in 1TL is probably associated with increased iron needs induced by strenuous training stimulus. Hepcidin increase at the highest training intensity point (2TL), probably reflects cumulative effect of prolong training result from-exercise induced inflammation. In recovery process (BPost), hepcidin decrease could be explained by increased iron needs, caused by increased turnover of red blood cells.

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PULMONARY HYPERTENSION IN YOUNG CHILDREN WITH SICKLE CELL DISEASE: WHEN DOES IT START? SURVEY AMONG IMMIGRANT PATIENTS IN NORTH-EAST ITALY

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Background. Acute Chest Syndrome (ACS) and Pulmonary Hypertension (PH) are among the leading causes of death in adults with Sickle Cell Disease (SCD). Several studies have been conducted in children utilizing a Tricuspid Regurgitant Velocity (TRV) TRV≥2.5 m/sec as a marker of PH. Despite the finding of elevated TRV in children below 5 years of age, the prevalence, age at onset and evolution of PH in children with SCD are still not clear. Evaluation of TRV has been performed mainly in African-Americans and mean age of analyzed populations is above 10 years. It is well known that SCD displays phenotypic variability among populations and individuals and that vascular complications may vary in different ethnic groups. In Italy, like in the rest of Europe, SCD patients are mainly African immigrants and they are very young. *Aims.* To determine prevalence and natural evolution of elevated TRV in a population of young African patients with SCD, mainly treatment free. To identify social, clinical, laboratory or echocardiographic predictive factors for precocious development of PH in children. *Methods.* Since 2007 we performed annual echocardiography with evaluation of TRV and Tissue Doppler Imaging (TDI) to SCD children ≥3 years of age at steady state and to racially/age-matched controls. Clinical, laboratory and echocardiographic variables were analysed to identify predictive factors for early development of PH. Patients receiving chronic transfusion were excluded.

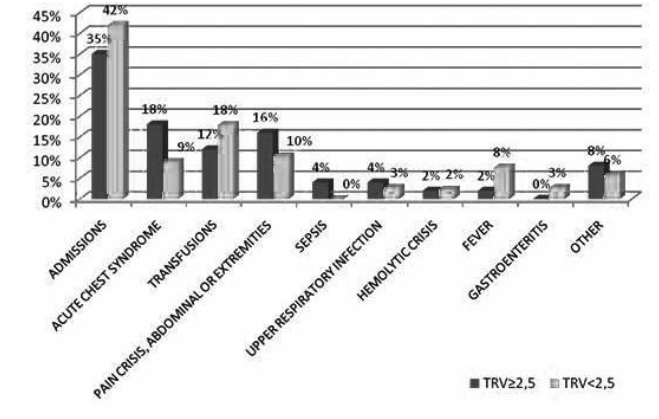

Figure.

Results. 41/75 patients were ≥ 3 years and 37 had measurable TRV. Mean age was 6.2 years (range 3,07-15,68), 86% were Africans, 85% were HbS/HbS. 8/37 (21.6%) had TRV≥2.5 m/sec, three of them being only 3 yrs old. Three patients were on hydroxyurea, all in the TRV<2.5 m/sec group. Patients with higher TRV displayed higher platelet (P=0.017) and reticulocyte counts (P=0.033) and a higher mitral E/A ratio (P=0.024). They had higher Blood Pressure (50% with BP>90th percentile *vs.* 35%) and more ACS (18 *vs.* 9%), even if steady state Oxygen Saturation remained normal (97% *vs.* 98%). In the multivariate analysis only ACS remained as a predictive factor for development of high TRV (P=0.035). After 3 years of follow-up all patients in the TRV ≥2.5 m/sec group had further elevation of the TRV, with the overall prevalence of PH in the population increasing to 40,5%. TDI revealed initial signs of diastolic dysfunction of the left ventricle (LV) as demonstrated by higher late diastolic velocities. At follow-up, TRV patients showed a progressive dilatation of the LV, probably related to their chronic volume overload. *Conclusions.* Our study suggests that elevation of TRV and diastolic dysfunction of the LV in SCD children begin earlier than previously expected. Factors leading to early onset of PH in children might not exactly be the same as the ones causing its development in older children or adults. Studies with longer a follow-up and larger population are warranted in order to better define significance of elevated TRV in childhood and to evaluate the steps leading to cardiac dysfunction. Nevertheless, it seems that African children with SCD might benefit from early screening and yearly re-assessment.

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CHARACTERIZATION OF ALPHA-THALASSEMIC GENOTYPES BY MLPA (MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION)

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Background. Alpha (α) thalassemia is an inherited hemoglobin disorder characterized by a quantitative reduction of the α-globin chains. Genomic deletions involving the α-globin gene cluster on chromosome 16p13.3 result in one or more α-globin genes being missing and are the most common molecular cause of α-thalassemia. The molecular test commonly used to identify these deletions is gap-PCR, which can only be used to detect known deletions in which both breakpoint sequences have been previously characterized. Recently, a simple technique suitable for rapid quantitative analysis called Multiplex Ligation-dependent Probe Amplification (MLPA) has been described. *Aims.* We describe here eight deletions (α0) and one segmental duplication involving the α-globin gene cluster in unrelated individuals using the MLPA method. *Patients and Methods.* Approximately 38 to 57 probes were used; these had lengths between 90 and 409bp and were distributed along a 700kb genomic region from the tip of the short arm of chromosome 16. Five of the patients had HbH disease and were found to have both the α0 and common α+3.7 deletions, the latter of which had already been identified by gap-PCR; three cases were α0 heterozygotes and in one the MLPA analysis revealed a full duplication of the α-cluster, extensive to the chromosome 16p telomer. *Results.* Four of the deletions found are large and remove the complete α-cluster and its upstream regulatory element (HS-40). These deletions span at least a genomic region of 270kb, 450kb, 540kb and 760kb (positions 43625-288064, 43625-583598, 43625-583598 and 43625-756403 of the UCSC Genome Browser, respectively). Another four deletions were limited to a region that contains the HS-40 element, leaving the α-globin genes intact but without expression. These deletions span a region of at least 60kb in the first case and 140kb in the others (positions 75563 - 132952, 43625-142344, 43625-152736 and 43625-152736 of the UCSC Genome Browser, respectively). Finally, in the case with α-cluster duplication, the duplicated allele spans at least 170kb (position 43625-175806 of the UCSC Genome Browser). *Conclusion.* These results show that MLPA is a suitable method for detecting unknown and uncommon deletions and duplications. Although breakpoints have not been determined yet, most of the deletions seem to be novel. Financial Support: FAPESP, CNPQ and CAPES / Brazil.

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EVALUATION OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA DISEASE BURDEN IN PATIENTS ENROLLED IN THE INTERNATIONAL PNH REGISTRY

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Background. Paroxysmal nocturnal hemoglobinuria (PNH) is a rare clonal hematopoietic stem cell disease which can lead to life-threatening complications including thrombotic events (TE), kidney disease, and pulmonary hypertension. Greater use of high sensitivity diagnostic tests and the availability of targeted terminal complement blockade treatment have lead to an increase in PNH disease awareness and improved prognosis, respectively. We have established an international PNH Registry in order to assess disease burden and examine the natural history of PNH. *Aims.* To describe the spectrum of disease burden assessed by patient-reported symptoms, history of thrombosis (TE), and PNH granulocyte clones size (PNH clone), together with evaluation of co-existent bone marrow disorders (BMD). *Methods.* Data from 78 clinical sites in 14 countries on 4 continents (as of February 2010) was analyzed to determine symptom presentation of patients with PNH. Sites collected disease-specific data including demographics, laboratory values, PNH clone size, medical history, symptoms, TE, and treatments. *Results.* As of February 2010 there were 524 enrolled patients in the Registry. Mean age at enrollment was 44±17 (range 5-89) and 54% were female. Median PNH clone size was 80% (15% of patients with PNH clone <10% and 70% with clone ≥50%). History of BMD was noted in 42% of patients (37% aplastic anemia [AA], 5% other). 74% of patients with AA had PNH clones >10% compared to 93% without any BMD (P<.01). The mean LDH was 1.2 times the normal upper limit in patients with PNH clones <10%, 2.5 times with clones 10-49%, and 4.3 times with clones ≥ 50% (P<.01). LDH was similar in patients with and without a history of BMD (3.1 and 3.7 times the normal upper limit, P=0.33). Mean age at first symptoms was 36±17 (range 5-79). At study enrollment, 46% of patients reported abdominal pain, 58% reported shortness of breath, 28% reported chest pain, 76% reported fatigue and 65% reported discolored urine. PNH symptoms were present regardless of PNH clone size (Table). Additionally, patients with underlying AA demonstrated symptoms at equal frequency to patients with no history of BMD. Patients with PNH clone >50% were more likely to have a history of thrombotic events (22%) compared to patients with smaller PNH clones, however the risk of TE is still high in patients with PNH clones <10% (5%) over the general population. Less than half the patients entering the registry required transfusions in the year prior to enrollment (38%). Other treatments that patients received in the prior year were anticoagulants (32%), eculizumab (28%), and immunosuppressive therapies (21%).

Table. Symptoms and complications of PNH.

	PNH Clone				Bone Marrow Disorder		
Symptom or complication	<10%	10-49%	>50%	P-value*	Aplastic or Hypoplastic Anemia	No Bone Marrow Disorder	P-value**
TE	5%	5%	22%	<.01	18%	19%	0.89
Abdominal Pain	41%	53%	46%	0.58	48%	47%	0.92
Shortness of breath	49%	44%	53%	0.63	53%	61%	0.23
Chest pain	14%	31%	24%	0.21	31%	27%	0.43
Fatigue	59%	72%	76%	0.15	75%	76%	0.99
Discolored urine	30%	56%	75%	<.01	60%	72%	0.05

*Chi-square test for differences across three PNH clone size categories.
** Chi-square test for differences across two BMD categories.

Conclusions. Clinical presentation at study enrollment showed that abdominal and chest pain, shortness of breath, fatigue, and discolored urine is prevalent in PNH patients. PNH symptoms were reported in patients irrespective of large or small clones, including evidence of a clinical TE, or history of BMD. This global PNH Registry will continue to evaluate disease burden and determine the long-term natural history of PNH and treatments outcomes. New clinical sites and geographic regions are encouraged to participate in the Registry.

1023

FOK-I GENE POLYMORPHISM OF VITAMIN D RECEPTOR IN PATIENTS WITH β-THALASSAEMIA MAJOR. ASSOCIATION WITH LEVELS OF VITAMIN D METABOLITES

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Background. Vitamin D deficiency is increasingly identified among patients with β-thalassaemia, whereas recent evidence associates it with increased myocardial iron accumulation. Most of the biological actions of vitamin D are mediated by an intracellular receptor (VDR) in whom several single nucleotide gene polymorphisms, that could potentially modify its action, have been identified. *Aims.* The aim of the study was to assess the distribution of Fok-I genotype of VDR among Greek children and young adults with β-thalassaemia major and consecutively to investigate its association with levels of $25(OH)D_3$ and $1,25(OH)_2D_3$. *Methods.* Blood samples from 69 patients with β-thalassaemia major on conventional treatment (35 females and 34 males), with a mean decimal age of 23.05±6.07 years, were collected for the determination of $25(OH)D_3$ and $1,25(OH)_2D_3$ serum concentrations and the genotyping of Fok-I polymorphism in exon 2 of the VDR gene on chromosome 12. *Results.* With regard to Fok-I polymorphism, results showed no deviation from Hardy-Weinberg equilibrium as 44.9% of the patients were homozygotes for F allele, whereas homozygosis for f allele and heterozygosis (Ff) were presented in 11.6% and 43.5% of the patients respectively. Markedly decreased levels of serum $25(OH)D_3$ were observed as 41 patients (59.4%) were below the cut-off limit of 50 nmol/l that determines deficiency, whereas, levels of $1,25(OH)_2D_3$ showed a wide variability ranging from deficiency (≤ 50 pmol/L) in 34 patients (49.3%) to excess (≥ 125 pmol/L) in 13 patients (18.8%). When patients were stratified according to their $1,25(OH)_2D_3$ status, a higher prevalence of f allele was recorded in the insufficiency group (P=0.03, Figure). Finally, levels of the two metabolites of vitamin D showed a negative correlation, approaching statistical significance (r=-0.204, P=0.09). *Summary/Conclusions.* Our study showed significantly decreased levels of 25(OH)D3 in patients with β-thalassaemia major and an association between levels of 1,25(OH)2D3 and the presence of f allele of the Fok-I polymorphism. More studies are required to further investigate the genetic contribution to the regulation of vitamin D metabolites as their deficiency has been lately associated with unfavorable sequelae in patients with β-thalassaemia major.

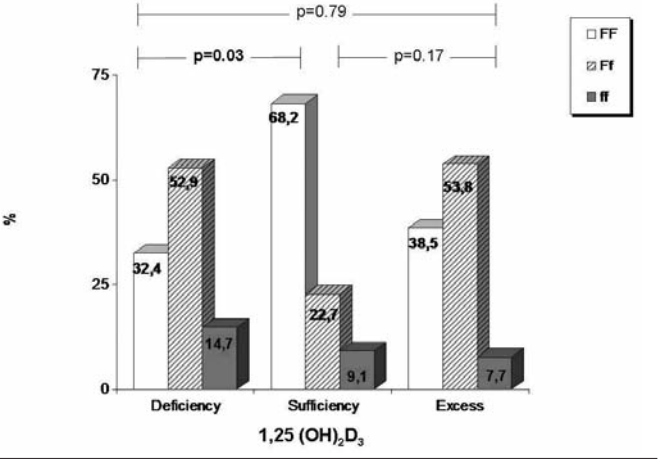

Figure. Higher prevalence of f allele in vitD deficiency.

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SICKLE CELL DISEASE: FROM NEONATAL SCREENING TO CLINICAL MANAGEMENT

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A neonatal haemoglobinopathy screening programme was implemented in Brussels more than a decade ago and in Liège five years ago, the programme being adapted to the local situation. It is universal, performed using liquid cord blood and an isoelectric focusing technique. All samples with abnormalities undergo confirmatory testing. Major and minor haemoglobinopathies are reported. Affected children are referred to a specialist centre. A central data base in which all screening results are stored are available and accessible to local care workers. A central clinical database to monitor follow-up is under construction. The objectives of the clinical data base are learning the characteristics of this population, creating a network between practionners (general, emergency and specialist practionners), State-of-the art, Guidelines, a tool for disseminating information, a tool for education projects, a basis for research, and could represent a pilot project for other chronic diseases. Since 1994, around 250,000 newborns were screened. The annual incidence of sickle cell disease is±1/1500, of β thalassaemia major and haemoglobin H disease is <1/25,000. All major haemoglobinopathies were confirmed and follow-up of the infants was undertaken except for three of them who did not attend the first medical consultation despite our best efforts. The clinical data base, approved by local ethical committee and with the informed consent of each patient, contains actually 280 patient records. The first information provided are the predominance of patients from DR Congo (67.5%), the high amount of severe clinical events (85%, the predominance of Hb SS phenotype in number and severity (90%), the report of severe infections which remain still very worrying (8.2%), but also that clinical and radiological neurological events are sizeable, and that there is a good response to treatment intensification, particularly to BMT and hydroxyurea. Of course it raises also many questions. In conclusion, the universal neonatal screening programme was effective because no case of major haemoglobinopathy was reported to be unidentified. The affected children received dedicated medical care from birth. The screening programme and specifically the reporting of minor haemoglobinopathies has been an excellent health education tool in Belgium for more than 12 years. On the other hand, the clinical database is important to improve clinical management of patients with SCD. It is a possibility to identify risk factors and tailor treatments; it constitutes a basis for prospective studies to valid criteria of disease severity and to adopt guidelines for adequate treatment.

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PHOSPHOGLYCERATE KINASE DEFICIENCY: FUNCTIONAL PROPERTIES OF THE L89P, K191DEL, C316R AND A354P PATHOLOGICAL VARIANTS

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Background. Phosphoglycerate kinase 1, (PGK1) the isoenzyme that catalyzes the first ATP-generating step in the glycolytic pathway, is encoded by a gene located on the X-chromosome, and is ubiquitously expressed. It is a relatively small monomeric molecule characterized by two hinge-bent domains, with a highly conserved structure. The N-terminal domain binds 1,3-bisphosphoglycerate (1,3BPG) or 3-phosphoglycerate (3PG), whereas the C-terminal domain binds Mg-ADP or Mg-ATP. During the catalytic cycle, the enzyme undergoes large conformational rearrangements, proceeding from an open form to a closed form. Mutations of the PGK1 gene result in an enzyme deficiency that is characterized by mild to severe haemolytic anaemia, neurological dysfunctions and myopathy. Patients rarely exhibit all the three clinical features. To date, 20 different mutations with worldwide distribution have been described. *Aims.* To gain knowledge on molecular bases of the haemolytic anaemia and/or neurological and muscolar dysfunctions associated to PGK deficiency, an in depth characterization of the PGK variants has been undertaken. The present study describes the biochemical characterization of L89P (c.266T>C), K191del (delAAG c.571>573), C316R (c.946T>C) and A354P (c.1060G>C) variant enzymes. K191del mutation was found in a patient affected by haemolytic anaemia; L89P, C316R and A354P mutations were identified in patients with haemolytic anaemia and neurological dysfunctions. *Methods.* Site-directed mutagenesis was used to introduce mutations into the PGK cDNA. All variants were expressed as recombinant forms in a microbial system and purified to homogeneity after a single anion exchange chromatographic step. *Results.* All mutations turned out to have detrimental effects on the molecular properties of PGK enzyme. All variants displayed high heat instability, being L89P, C316R and A354P the most affected (T50 values approximately 10°C lower than that of the wild type enzyme; t1/2 at 37°C, 9, 12 and 30 min, respectively, *vs.* >2h of the wild type). Differently from the wild type enzyme, all variants were not protected from heat inactivation by ATP or 3PG. In addition, A354P variant showed a Km value *vs.* 3PG 15-fold higher than that of the wild type enzyme, accounting for a 18-fold lowered catalytic efficiency. K191del and C316R enzymes exhibited 6- and 4-fold reduced kcat values. *Conclusions.* On the whole, these data primarily indicate that all mutations affect residues crucial for protein stability. Most probably, the inability to maintain proper folding *in vivo* makes the enzymes good targets for degradation by the ubiquitin-proteasome pathway, thus reducing their intracellular concentration, mainly in red blood cells. Moreover, the altered kinetic properties displayed by K191del, C316R and A354P variants render these enzymes unable to fulfil properly the catalytic cycle, contributing to lower the ATP generation.

1026

REGIONAL AND GLOBAL PANCREATIC T2* MRI FOR IRON OVERLOAD ASSESSMENT IN A LARGE COHORT OF HEALTHY SUBJECTS: NORMAL VALUES AND CORRELATION WITH AGE AND GENDER

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Background. Impairment of the endocrine and exocrine function of the pancreas is a common complication in patients with systemic iron overload, especially in thalassemia patients. Multiecho T2* MRI is a well-established technique for iron overload assessment but there are few reports concerning the pancreas. *Aims.* Our aims were to assess the feasibility and reproducibility of the MRI technique for measuring pancreatic regional and global T2* values, to establish the lower limit of normal in a large cohort of healthy subjects and to correlate the gained values with age and gender. *Methods.* One hundred and twenty healthy subjects (61 males, mean age 51±17 years) underwent MRI exam (1.5T) using a breath-hold multiecho T2* gradient-echo sequence. T2* measurements were performed in pancreatic head, body and tail. The global value was calculated as the mean.

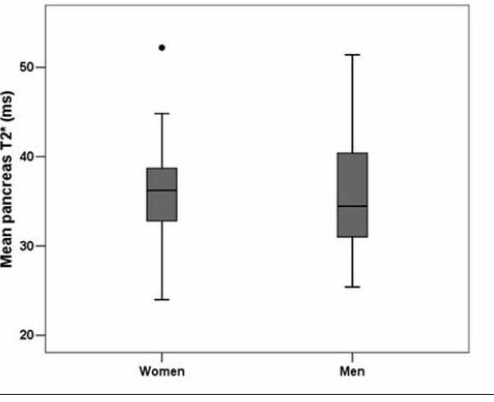

Figure.

Results. Measurement of pancreatic T2* values was feasible in all subjects and in 86% it was possible to perform measurements in each region. For the T2* global value the CoV for intra-operator and inter-operator reproducibility were 7.7% and 13%, respectively. The global T2* value ranged from 24 to 52 ms with the lower limit of normal of 26 ms. There were no significant differences among the regional pancreatic T2* values. No significant correlation was found between T2* and patient age or gender. *Conclusions.* In conclusion, pancreatic T2* measurements appear to be feasible, reproducible, non-time-consuming and reliable. In a large cohort of healthy subjects gender- and age-related differences concerning pancreatic T2* were not found.

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SIRES: A WEB-SERVER TOOL FOR SEARCHING OF IRON-RESPONSIVE ELEMENTS

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The IRP/IRE regulatory system plays a crucial role in the post-transcriptional regulation of gene expression and its disruption results in human disease. Iron-responsive elements (IREs) are cis-acting regulatory motifs present in mRNAs that encode for proteins involved in iron metabolism. They function as binding sites for two related trans-acting factors, namely the iron regulatory proteins (IRP1 and IRP2). Among the cis-acting oligonucleotide patterns, the IRE is one of the best characterized. It is defined by a combination of RNA sequence and structure. However, currently available programs to predict IREs do not show a satisfactory level of sensitivity and fail to detect functional IREs. Here, we report an improved software for the prediction of IREs implemented as a user-friendly web-server tool. The SIREs web-server uses a simple data input interface and provides structure analysis, predicted RNA folds, folding energy data and an overall quality flag based on properties of well characterized IREs. Results are reported in a tabular format and in a schematic visual representation that highlights important features of the IRE. The SIREs (Search for iron-responsive elements) web-server is freely available on the web at http://ccbg.imppc.org/sires/index.html.

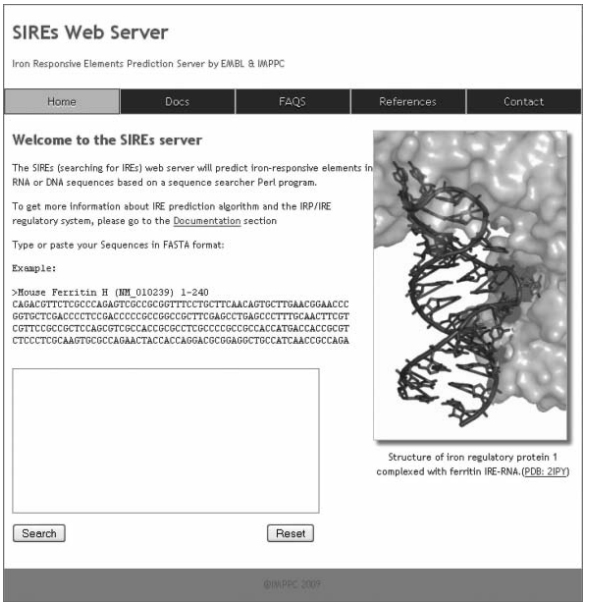

Figure. SIREs web-server input page.

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CENTRAL NERVOUS SYSTEM FINDINGS IN YOUNG PATIENTS WITH THALASSEMIA INTERMEDIA

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Background. Several reports have demonstrated involvement of the nervous system in thalassemia patients, with a high incidence of thromboembolic events especially in thalassemia intermedia (TI) patients. Brain involvement has not been widely studied in TI, although limited reports confirm a low incidence of stroke and high incidence of silent brain infracts. In most cases, neurological involvement does not initially present with relevant signs or symptoms and can only be detected during neurophysiological or neuroimaging evaluation. *Aim.* To evaluate possible CNS involvement in young patients with thalassemia intermedia. *Methods.* 19 patients with thalassemia intermedia , 12 females and 7 males, with a mean age of 13 years (range: 8-20 years) participated in a systemic neurophysiologic and neuroimaging study. None of them were on a regular transfusion program or on chelation therapy. Neurological examination was carried out and hemoglobin and serum ferritin were measured in every patient. Neurophysiological evaluation consisted of electroencephalogram (EEG), brainstem auditory (BAEP), visual (VEP) and somatosensory (SEP) evoked potentials examination, as well as, transcranial Doppler (TCD) ultrasonography, magnetic resonance imaging (MRI) and angiography (MRA) of the brain. *Results.* None of the patients reported symptoms of overt stroke and none demonstrated abnormal neurological findings on clinical examination. From the neuroimaging evaluation none of the patients had abnormal findings on MRI or MRA of the brain. The blood flow velocity in the basal intracranial arteries was normal for all patients. There was 1/19 (5.2%) patient with prolonged latencies in BAEP examination. VEP and SEP examinations were normal in all patients. 8/19 (42%) patients had abnormal findings in EEG, consisting of diffused slow waves (21%), slow waves mixed with sharp waves (15.7%) and paroxysmal atypical spikes (10.5%). No correlations were found between EEG findings and age, gender, hemoglobin or serum ferritin levels. *Conclusions.* The results of our study show the presence of neurophysiological abnormalities even in young patients with thalassemia intermedia. The EEG findings are probably attributed to the anemia dependent chronic hypoxia, as suggested previously by other authors in thalassemia major groups of patients. There were no abnormal MRI-MRA findings, probably because of the young age of our group, as other studies have demonstrated brain lesions in adult TI patients and increasing age has been associated with higher incidence and multiplicity of events.

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THE IMPACT OF RECENTLY-PUBLISHED NEGATIVE ERYTHROPOIETIN STIMULATING AGENTS (ESA) STUDIES ON CLINICAL MANAGEMENT OF CANCER RELATED ANEMIA. A COMPREHENSIVE CANCER CENTER EXPERIENCE

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Anemia is the most common hematological abnormality in cancer patients. Unfortunately, it is often under-recognized and under-treated. Erythropoietin stimulating agents (ESA) were widely used to prevent and treat cancer and chemotherapy-related anemia. However, many recent studies suggested a negative impact on disease progression and survival associated with their use. This study describes the prevalence of anemia in cancer patients and the recent trends in its management given the recent negative studies. A total of 959 patients were included, the mean age (SD) was 53 (16) years, 492 (51%) of the patients were males. Main reasons for admission at time of enrollment were chemotherapy in 217 (22.6%), infections including neutropenic fever in 190 (19.8%), palliative and supportive care in 145 (15.1%). Primary cancer diagnoses were: gastrointestinal in 203 (21.2%), breast in 154 (16.1%), lymphoma/multiple myeloma in 151 (15.7%), and lung in 106 (11.1%). At time of enrollment, anemia (Hb <12 g/dL) was detected in 755 (78.7%) patients. Mean Hb value for anemic patients was 9.5 g/dL (range 3.5-11.9, median 9.6). Severe anemia (Hb <8.0 g/dL) was detect-

ed in 126 (16.7%), moderate (Hb: 8.0-9.9 g/dL) in 319 (42.3%) and mild (Hb: 10-11.9 g/dL) in 310 (41.1%) patients. Prevalence and severity of anemia varied according to tumor type and reason for admission. More than two thirds [518 (68.6%)] of the anemic patients were not offered any kind of anemia treatment, however, most (60%) of them had mild anemia. Mean Hb value at which treatment was started was 8.0 g/dL, while mean Hb for the patients who were not treated was 10.2 g/dL. Anemia treatment was related to its severity; 119 (94.4%) of the patients with severe anemia were treated compared to a treatment rate of 32.9% in the group with moderate anemia, and 5.0% in patients with mild anemia (P<0.0001). Treatment offered for patients with anemia varied; blood transfusion was given to 188 (79.3%), while ESA were offered for only 8 (3.4%) of the treated patients. Treatment rate also varied according to reason for admission; only 29 (21.6%) of anemic patients admitted for chemotherapy were offered any treatment compared to a rate of 33.4% for the rest of the cancer patients, (P=0.0075). Length of hospital stay was affected by the presence of anemia; the mean hospital stay was 7.27 [95% CI: (6.95, 7.59)] days in anemic patients compared to 4.85 [95% CI: (4.59, 5.11)] days among the non-anemics (P<0.001). In addition to anemia status; age, reason for admission, primary tumor site and stage were studied for impact on length of hospital stay; primary disease (P<0.0001) and reason for admission (P=0.053) were associated with significant impact. *Conclusions.* Given the recent negative ESA studies and FDA warnings, anemia is under-recognized and under-treated. Only severe anemia was adequately treated; mostly with blood transfusion while ESA were rarely used. Majority of patients with moderate anemia were not treated including patients on active chemotherapy. Better guidelines addressing anemia management in this subgroup of patients are highly needed.

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THE EFFECT OF HELICOBACTER PYLORI INFECTION ON SERUM VITAMIN B12 AND HOLOTRANSCOBALAMIN LEVELS IN CHILDREN

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Background. Helicobacter pylori (H pylori) is a gram negative bacterium that has been found to be associated with vitamin B12 deficiency. Vitamin B12 is required by all cells for its role in one-carbon metabolism and in DNA-synthesis and maintenance. The clinical consequences of vitamin B12 deficiency include megaloblastic anemia and progressive neurologic disease of the central and peripheral nervous systems. Early diagnosis of vitamin B12 deficiency is crucial because of the possible risk of irreversible neurological damage. Holotranscobalamin assay is considered as a convenient approach that measures the active portion of B12 and the only part available for the cell-use. *Aims.* The purpose of this study was to evaluate the association between serum vitamin B12 levels and H pylori infection and examine the clinical usefulness of holotranscobalamin measurement in children. *Methods.* A total of 30 children, who suffered from abdominal pain, nausea, vomiting or lack of appetite and diagnosed to have H. pylori infection by C-14 urea breath test, constituted the study group. The control group consisted of 26 children without H. pylori infection. In both the study (before and after treatment) and the control groups, complete blood count (CBC), creatinine, vitamin B12, folate, plasma total homocystein, and holotranscobalamin measurements were performed. The children in the study group were given a 2- week course of oral proton pump inhibitor plus Amoxicilline plus Clarithromycine therapy. The study was approved by the local ethics committee. *Results.* There were no statistically significant differences between the study and control groups with respect to age and gender. The study group pre-treatment and post-treatment parameters and control group parameters did not differ significantly in values for CBC, creatinine, vitamin B12, folate and plasma total homocystein. In the study group, the pretreatment plasma holotranscobalamin concentrations were significantly lower than post-treatment (P<0.02) and those in the control group (P<0.05). No statistically significant differences were found between the study group post-treatment values and the control group with respect to holotranscobalamin. *Conclusions.* Direct measurement of holotranscobalamin has been postulated to provide a better indicator of an individual's vitamin B12 status. The measurement of total serum vitamin B12 suffers from a number of limitations, most particularly that the majority of vitamin B12 measured is that bound to haptocorrin. Given that holotranscobalamin has a shorter circulating half-life than holohaptocorrin, the earliest change that occurs when an individual enters a negative vitamin B12 balance is very likely to be a decrease in the plasma holotranscobalamin concentration. In this study, we demonstrated that H pylori infection in children may significantly decrease plasma holotranscobalamin concentrations, which can be reversed by eradication therapy. The findings of our study suggest that H pylori infection has a negative effect on vitamin B12 levels.

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RELATIONSHIP BETWEEN GDF-15 (GROWTH DIFFERENTIATION FACTOR 15) AND IRON OVERLOAD IN THALASSEMIA INTERMEDIA PATIENTS

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Introduction. Thalassemia intermedia (TI) encloses a wide spectrum of transfusion independent thalassemias, nevertheless patients have iron loading due to increased intestinal iron absorption and ineffective erythropoiesis. Accumulation of iron in absence of blood transfusions may result from inappropriate suppression of hepcidin by high erythropoietic activity that overwhelms iron load. Hepcidin is a small liver peptide that reduces iron absorption via degradation of ferroportin. GDF-15 is a divergent member of the transforming growth factor-_ superfamily identified as a hypoxia-inducible gene product involved in hepcidin regulation. Recent reports have described GDF-15-dependent suppression of hepcidin in β-thalassemia patients. *Aim.* To evaluate if GDF-15 relates with iron status in thalassemia, serum ferritin (SF), transferrin saturation (TS%) and NTBI were determined in 40 TI patients (aged 43±11; 28 splenectomized and 12 occasionally transfused), in 12 TI very mild due to triplicated α genes plus β mutation (β°/ααα) aged 43±13 years and in 20 healthy subjects as controls. *Methods.* Serum GDF-15 was evaluated by enzyme-linked immunosorbent assay using DuoSet Sandwich ELISA Kit (R&D Systems, Minneapolis, MN); serum hepcidin was measured by ELISA Kit (DRG Instruments GmbH, Germany). NTBI was assayed by HPLC after nitrilotriacetic acid chelation. *Results.* GDF-15, hepcidin and NTBI values were significantly higher in TI (23567± 17911 pg/mL; 9.71±8.58 ng/mL; 1.47±2.26 μM respectively) compared to controls (581±242 pg/mL, P<0.0001; 3.5±2.5 ng/mL, P=0.05; -0.72±0.70 μM, P=0.05 respectively). In splenectomized patients GDF-15 levels were higher although non significant than in non splenectomized (26730±15852 pg/mL *vs.* 22785±18331 pg/mL), hepcidin was lower (7.6 ± 5.8 ng/mL *vs.* 11.4±10.9 ng/mL) and NTBI was increased (2.02±2.29 μM; 0.51±2.0 μM respectively). In transfused patients GDF-15 and NTBI were significantly higher than in non transfused (P=0.05; P=0.015 respectively), whereas hepcidin levels, although higher, were not significant. GDF-15 showed a significant positive correlation with SF in transfused and splenectomized patients (r=0.766 P=0.0061; r=0.521 P=0.05). In subjects with β°/ααα GDF-15, hepcidin and NTBI were significantly lower (4085±2011 pg/mL, P=0.018; 4.8±2.0 ng/mL, P=0.015; 0.13 ±1.61 μM, P=0.023 respectively) compared to other forms of TI. *Conclusions.* Our results confirmed that in TI iron regulation is also mediated by high concentrations of GDF-15 related to ineffective erythropoiesis, tissue hypoxia, and erythroblasts apoptosis. In fact by our study GDF-15 production affecting consequently hepcidin synthesis seems related to iron overload in splenectomized and transfused patients. In mild forms of TI, such as β°/ααα, GDF-15, hepcidin and NTBI are slightly affected.

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PULSED DEXAMETHASONE FOR THE TREATMENT OF PATIENTS WITH AUTOIMMUNE HEMOLYTIC ANEMIA (SINGLE CENTER EXPERIENCE)

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Background. Steroid administration is still first-line therapy for autoimmune hemolytic anemia (AIHA). The activity of high dose dexamethasone has been largely investigated in immune thrombocytopenic purpura, and in AIHA. *Aims.* The aim of this study is to evaluate the therapeutic results and side effects of dexamethasone pulse therapy in AIHA patients in the Hematology Clinic of Timisoara. *Methods.* Between I 2007 and XII 2009, 35 patients with a newly diagnosed AIHA (23 cases) or a first relapse of AIHA (12 cases) were treated. The median age of patients was 54 years (range: 26-74), 22 patients were females and 13 males. The immunohematologic study revealed an anti-erythrocyte antibody (AeAb) in all cases (IgG: 26; IgM:6; IgG + IgM:1; IgA:1;

IgG+IgA:1). An idiopathic AIHA was diagnosed in 25 cases (71,4%), while in 10 (28,5%) a concurrent disease was identified. (tumor: 3; autoimmune disease: 3; lymphoproliferative disease: 4 cases). Treatment consisted of 4-6 courses of dexamethasone 20 mg, total dose, administered i.v. for 5 consecutive days, every 4 weeks. Between each dexamethasone course, patients received prednisone orally at the initial dose of 0.5 mg/kg/day. During the subsequent course, prednisone was slowly tapered by 5 mg every 1-2 weeks to a minimum dose of 10 mg 3 times a week in patients who reached a Hb value ≥12 g/dL. After maximum 6 courses of dexamethasone, response was assessed according to the Hb value combined with the evaluation of the direct antiglobulin test (DAT). Patients with no detectable AeAb and persistent Hb values ≥ 12 g/dL were considered as complete responders (CR) and steroid treatment withdrawn. Patients with persistent AeAb, but with a Hb increase of at least 3 g/dL, were considered as partial responders (PR). 2-3 further dexamethasone courses were given to PR patients, while patients with a persistent Hb value ≥ 12g/dL underwent a maintenance dose of 10 mg three times a week. All patients received folic acid supplementation, trimethoprimcotrimoxazole as Pneumocystis carinii prophylaxis and proton pump inhibitors as prophylaxis of related gastritis. Red cells were infused in the presence of severe and symptomatic anemia. *Results.* Response was assessed in 30 cases. The OR was 83% (CR:15%; PR: 68%) was similar in previously treated and untreated patients. A hemolytic relapse was observed in 3 cases. The median duration of response of patients with PR and maintained with low doses of prednisone was 23 months (range: 3-44), and 36 months, respectively, in patients with idiopathic AIHA who achieved a CR. No significant side effects were recorded. *Conclusions.* Pulsed dexamethasone therapy is effective and well tolerated in AHA patients. CR is rare. Additional studies need to be designed to explore new dexamethasone schedules and another treatment approaches such as rituximab.

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A T2* MRI PROSPECTIVE SURVEY ON HEART AND LIVER IRON IN THALASSEMIA MAJOR PATIENTS TREATED WITH DEFERASIROX, DEFERIPRONE AND DESFERRIOXAMINE

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Background. Most deaths in thalassemia major (TM) result from cardiac complications due to iron overload. Available three iron chelation regimes in thalassaemia may achieve different changes in cardiac iron and function and liver iron, but to date no data are known in literature. Magnetic Resonance (MR) is the unique non invasive suitable technique to evaluated quantitatively this issue. *Aims.* The aim of this multi-centre study was to assess prospectively in the clinical practice the efficacy of the three available iron chelator regimes in monoterapy in a large cohort of TM patients by quantitative MR. *Methods.* Among the first 739 TM patients enrolled in the MIOT (Myocardial Iron Overload in Thalassemia) network, 253 patients performed a MR follow up study at 18±3 months according to the protocol. We evaluated prospectively the 140 TM patients who had been received one chelator alone between the 2 MR scans. We identified 3 groups of patients: 66 treated with desferrioxamine (DFO), 44 treated with deferasirox (DFX) and 30 treated with deferiprone (DFP). Myocardial and liver iron concentrations were measured by T2* multislice multiecho technique. Biventricular function parameters were quantitatively evaluated by cine images. *Results.* The dose of DFO was 41±7 mg/kg for 5.5 d/wk, DFX was 26±6 mg/kg/d and DFP was 73±16 mg/kg/d Excellent/good levels of compliance were similar in the 3 groups (DFO 92%, DFX 100%, DFP 97%; P =0.1). Among the patients with no significant myocardial iron overload at baseline (global heart T2*±20 ms), there were no significant differences in all 3 groups to maintain the patients without myocardial iron overload (DFO 100%; DFX 96%; DFP 100%; P=0.5). Among the patients with myocardial iron overload at baseline (global heart T2* <20 ms), in all 3 groups there was a significant improvement in the global heart T2* value (DFO P=0.003, DFX P=0.0001 and DFP P=0.001) and in the number of segment with a normal T2* value (DFO P=0.0001, DFX P=0.003 and DFP P=0.031); only in the DFO and DFP group there was a significant improvement in the right global systolic function (P = 0.045 and P = 0.031, respectively). The improvement in the global heart T2* was significantly greater for the DFP than the DFO and DFX group (mean difference global heart T2* 10.7±7.1 ms *vs.* 3.6±5.4 ms *vs.* 4.6±4.8 ms; P = 0.009) (Figure). The changes in the mean serum ferritin level and in the global systolic bi-ventricular function were not significantly different among groups. In patients with liver iron overload at baseline (liver T2* <5.1 ms), the change in the liver T2* was not significant among groups (mean difference liver T2* DFO 2.9±4.9 ms *vs.* DFX 2.4±5.2 ms *vs.* DFP 2.3±5.8 ms; P=0.95). *Conclusions.* Prospectively in a large clinical setting DFP monotherapy was significantly more effective than DFO and DFX over 15 months in improving myocardial siderosis in TM patients.

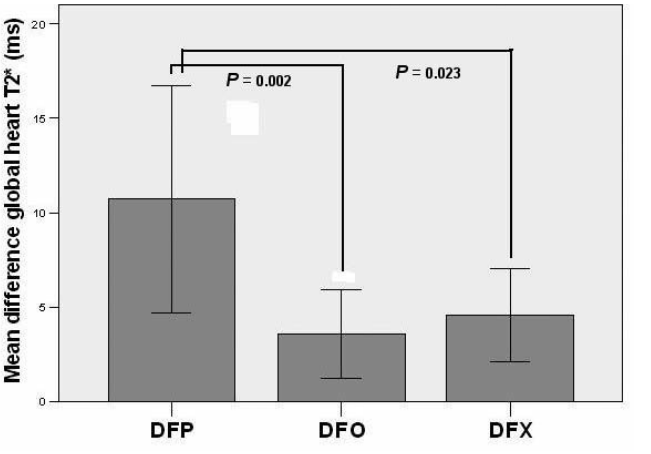

Figure.

Red cell/transfusion

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LIVER STIFFNESS MEASUREMENTS BY TRANSIENT ELASTOGRAPHY IN THALASSEMIA MAJOR

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The aim of this study was to investigate the correlation between liver stiffness, measured by transient elastography (TE) and liver iron overload markers with the use of magnetic resonance imaging (MRI) in patients with transfusion-dependent beta-thalassemia major. *Methods.* 91 patients with TM were included in the study. All patients were under chelation treatment with a pre-transfusion haemoglobin level of 9.7 g/dL. Liver stiffness measurements (LSM) were performed by the same operator, blinded to the results of the patients, using Fibroscan®, Echosens, Paris. The measurements were considered applicable if a success ratio of >60% and an interquantile range of <0.2 were achieved. Liver iron concentration (LIC) was evaluated by two magnetic resonance imaging methods (T2* and MRI.HIC) in 69/91 (76%) patients and by three methods (including R2) in 51/91 (56%) patients. MRI scans were acquired with an imager equipped with a 1.5 T magnet, and the data included hepatic iron measurements obtained by means of T2*. Additionally, calculation of MR-HIC values was based on an algorithm using liver to muscle (L/M) ratios in five axial gradient-echo sequences. Liver biopsy specimens were available in 16 patients. Hepatic inflammation and fibrosis were scored using the METAVIR score system. *Results.* The mean age of our patients was 28.2±8.6 years and 48 (53%) were females. The median values of liver tests were: AST= 25 IU/L (range 11-76), ALT= 24 IU/L (7-97), γgt= 20 IU/L (7-180), alkaline phosphatase= 80 IU/L (21-346). 69 (76%) patients were anti-HCV negative. The median ferritin level was 1010 ng/mL (100-7975). The applicability of LSM was 100%. The overall median LSM value was 6.8 kPa (4-40.3). LSM was higher in HCV positive patients (7.7 kPa *vs.* 6.6 kPa) when compared with HCV negative patients (P=0.04). Total LSM correlated adequately with liver tests (AST r=0,39 , P=0,0002 , ALT r=0,33 , P=0,0018, γGT r=0.44 , P<0,0001 and ALP r=0,26 , P=0,01) but showed no correlation wth serum ferritin (r=0.14 , P=0,18). LSM correlated weakly with LIC as assessed by MRI-T2* (ρ=-0.19, P=0.12), MRI-HIC (ρ=0.24, P=0.05) and MRI-R2 (ρ=0.22, P=0.13). However, these associations became statistically significant in the HCV negative group: MRI-T2* (ρ=-0.42, P=0.002), MRI-HIC (ρ=0.42, P=0.002) and MRI-R2 (ρ=0.46, P=0.003). Although the number of patients with liver biopsy was small, LSM seemed to correlate with the stage of fibrosis (5.9±1.5 for stage <2 *vs.* 8.1±2 for stage ≥2) as assessed in the biopsy (P=0.06). *Conclusions.* TE is a feasible alternative for the determination of liver fibrosis in TM patients. LSM seem to correlate with the histological findings and the HCV status of the patients. Additional data are warranted to clarify the relationship of LSM with LIC.

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HFE, HEMOJUVELIN, AND HEPCIDIN GENES SEQUENCING IN BRAZILIAN PATIENTS WITH PRIMARY IRON OVERLOAD

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Background. Low frequency of the *HFE* p.C282Y mutation was found in HH-affected Brazilian patients suggesting other HFE-related or HJV and HAMP gene mutations could be implicated in the pathogenesis or phenotypic expression of HH in our population. *Aims.* to evaluate mutations in HFE, HJV and HAMP genes and to identify the impact of HJV and HAMP mutations on HH phenotype in a possible digenic inheritance with HFE gene. *Methods.* Twenty-five Brazilian patients with primary iron overload (transferrin saturation >50% in females and 60% in males and absence of secondary causes) were included. Subsequent bidirectional sequencing for each HFE, HJV, HAMP exons and intron-exon regions was performed. The effect of HFE mutations on protein structure were analyzed by molecular dynamic and free energy calculations. Potential for change on the site of splicing were carried out. *Results.* Eighteen (72.0%) out of the 25 individuals presented at least one HFE mutation. Only 24.0% of patients carried 282YY genotype (n= 6) and 12.0% carried p.C282Y/p.H63D compound heterozygosity (n= 3). One novel mutation (*HFE* p.V256I) was indentified and the protein structural analysis showed that p.V256I mutation reduces the affinity binding between HFE and β2-microglobulin (β2M) in the same way that p.C282Y mutation when compared with the wild-type protein. HJV sequencing revealed a substitution in heterozygosis, c.929C > G, that corresponds to *HJV* p.A310G polymorphism; and in another individual, *HJV* IVS1 -36C > G intronic variant was detected in heterozygosis. In HAMP gene, one *HAMP* IVS3 +42G > A intronic variant was identified. *Conclusions.* The novel *HFE* p.V256I mutation reduces the interaction between HFE and β2M that may influence iron homeostasis, but further studies are necessary to investigate the effect of this mutation on HFE function and activity *in vivo*. *HJV* p.A310G polymorphism and two intronic variants were found, but none of these alterations were associated with digenic inheritance with HFE gene. Our data indicate that HJV and HAMP functional mutations are rare in these patients.

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THE RELEVANCE OF ANEMIA IN THE ELDERLY: A HOSPITAL-BASED ANALYSIS

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Background. Anemia represents a relevant challenge among the elderly as anemia is associated with decreased functional capacities and an increased mortality rate. *Aims.* To provide demographic data in a representative Middle-European population. To define the subtype and cause of anemia to form the basis for treatment algorithms. *Methods.* Laboratory values from 19758 patients older than 64 years treated at the Medical University Innsbruck, Austria from 1.10.2004 -29.9.2005 were analyzed. The cohort consisted of 10917 women and 8841 men, the median age was 75 yrs in women and 72 yrs in men (P<0.001). 10737 (54.3%) patients were treated on an outpatient basis, whereas 9.021 (45.7%) were inpatients. *Results.* Women revealed in general lower hemoglobin (Hb) values than men (median 13.4 *vs.* 14.3 g/dL; P<0.001). This phenomenon was not age-dependent as assessed by age-matched regression-analyses. Based on the WHO-criteria for the definition of anemia (Hb < 12 in female and < 13 g/dL in male), 19.3% of women and 23.4% of men suffered from anemia. The incidence of anemia was significantly correlated with advanced age as shown by correlation analysis (r=0.21; P<0.001). In the age groups 75-79, 80-84, 85-89, ≥ 90yrs, women were anemic in 20, 25, 29 and 33%, whereas men were anemic in 25, 34, 40 and 47% of cases, respectively. Based on the mean corpuscular volume of red blood cells, anemia was defined as microcytic in 3.7%, normocytic in 78% and macrocytic in 18.4% of cases. Microcytic and normocytic anemia were more frequent in women (58.2% and 52.5% of cases), whereas macrocytic anemie revealed a male preponderance of 58%. In microcytic anemia an iron deficiency as defined by lowered serum ferritin levels was observed in 73% and in 63.4% of cases (male/female). In macrocytic anemia lowered serum B12 levels were detected in 3% and decreased levels of folic acid in 7.4% of cases. In normocytic anemia C-reactive protein (CRP) was elevated in 64.7% and serum creatinine in 36.6%. Pronounced gender differences were observed, as elevations of creatinine were more often oberved in men (45%) than in women (28.4%). *Summary/Conclusions.* These data clearly demonstrate that late-life anemia is frequent in patients admitted to the hospital as well as in outpatients. Anemia increases dramatically with advanced age reaching a prevalence of nearly 50% in elderly men. A recognizable cause of anemia for which a specific treatment is available is found in a small but relevant proportion of patients. In the majority of elderly the pathogenesis of anemia is complex and includes a mixture of different subtypes including the anemia of chronic inflammation, of renal insufficiency and of decreased erythropoietin and androgen function.

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PRECISION OF MULTI-ECHO CMR FOR MYOCARDIAL IRON OVERLOAD EVALUATION IS DEPENDENT FROM MR SEQUENCE DESIGN

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Background. T2* multiecho CMR is largely used to assess iron overload in heart because of the established inverse relationship between the T2* value and the iron concentration in tissues. The decay of CMR signal is sampled at several echo times (TEs) and the T2* is inferred by fitting the decay curve to an appropriate model. *Aims.* Our aim was to quantify the reliance on TEs of the expected error in T2* value determination. *Methods.* The Cramer-Rao lower bounds theory (CRLB) was used. CRLB were evaluated for a commonly used multiecho sequence with the first TEs equal to the minimum achievable with the used scanner, echo spacing of 2.26 ms to minimize the fat-water interface artefacts, 10 echoes to assure acquisition in a single breath-hold. *Results.* Percent error in T2* values assessment was lower than 10% in the range of clinical interest, with the exception of very low T2* values. Precision in measurement of low T2* values is strongly dependent on the value of the first TE, that is limited by the used scanner. T2* values greater than 1.8 ms and 1.5 ms can be assessed with an error below 20% using a first TE of 2 ms and 1.5 ms, respectively (Figure). *Conclusions.* T2* multiecho sequences used in clinical practice assure an acceptable precision for T2* values greater than 2 ms or less, depending from the used hardware. For patients with very high myocardial iron overload sequences with lower minimum echo time and/or lower echoes interval may be useful.

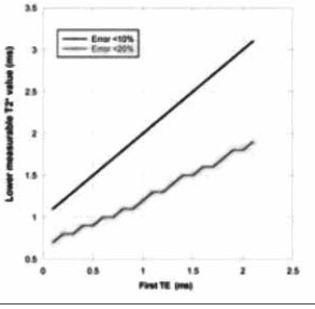

Figure.

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EPIDEMIOLOGICAL DATA OF HAEMOGLOBINOPATHIES AMONG IMMIGRANT POPULATION IN NORTHERN GREECE. A 15 YEARS REPORT

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Greece is a Mediterranean country with a high frequency of thalassaemia and haemoglobinopathies. Since 1993, an unprecedented wave of immigration of people of different backgrounds towards Greece and other states of the European Union has taken place. Consequently, the number of affected individuals and the spectrum of mutations have both increased and somehow changed. The immigrant population consisted mainly of people from Albania, the former Soviet Union, Africa, the Middle East and south East Asia. We report the results of haemoglobinopathy screening among this immigrant population during the period 1993-2007. *Methods.* The carrier identification was carried out by a standard scheme, which included, CBC and red cell indices using the Coulter ONYX, a Cation Exchange HPLC variant system (Bio-Rad, Variant β-Thalassaemia Short Program), to determine HbA, HbA2 and HbF levels and the different abnormal structural Hbs, electrophoretic techniques both at alkaline pH on cellulose acetate and at acid pH on citrate agar, sickling test and tests for HbH inclusion bodies. Haemoglobin A2 was also quantified by column micro chromatography and serum ferritin levels were measured by micro Elisa technique. Biosynthesis of the α- and β- globin chains and DNA techniques are also performed on selected cases. *Results.* From the total 50.194 subjects who underwent screening during this period, 5.399 (10.75%) were immigrants. 299 (5.5%) were carriers of thalassaemic genes or other haemoglobinopathies. The frequency of β-, α-, δβ-thalassaemia carriers was found to be 3.5 %, 0.8 %, and 0.16%, respectively. The prevalence of sickle cell carriers was 0.68 % and carriers for haemoglobinopathy Lepore (was) 0.16%. Two cases of haemoglobinopathy H and 3 cases of heterozygotes for HPFH were detected. Heterozygosities for HbO-Arab, HbC, HbE, Hb D were rare, and there was only one case from each group. Few cases of homozygotes and compound heterozygotes were encountered. These were β-thal/HbD- Punjab (1 case), homozygous β-thal. (1 case), α-thal/HbS (1 case), and HbS/HbC (1 case). Pertaining to their ethnic background, 32.5% were Albanians, 16.15 % from Pontos in Asia Minor, 13.7% from Georgia, and 6.1% were Albanian citizens of Greek origin who lived in South Albania, also known as North Epirus. Other minority groups were Armenians, Cypriots, Bulgarians, Serbians, citizens of the EU, Asia, Africa and Latin America, that consisted 15.1% of the total. During the last 7 years, 278 immigrant couples have been given genetic counselling in our Thalassaemia Prevention Unit. Knowledge of the epidemiology of haemoglobinopathies in this population group provides important information for public health planning and appropriate counselling of couples at risk of having an affected offspring. In addition, we conclude that, the spectrum of mutations has indeed been affected by the immigration of people of different ethnic backgrounds.

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MLPA CHARACTERIZATION OF THE (δβ)° SPANISH DELETION IN THALASSEMIC PATIENTS: A PROCEDURE FOR THE OPTIMIZATION OF DIAGNOSIS AND GENETIC COUNSELING

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Background. Nowadays, due mainly to the demographics changes have occurred in recent decades, the thalassemias are a group of emerging diseases in different European countries. Within this framework, both the genetic counseling and the prenatal diagnosis become crucial issues in order to minimize the effects in public health caused by these chronic diseases. The MLPA analysis allows, unlike other screening techniques, quick and easy identification of any copy number variation in the globin cluster. *Aim.* To characterize, by MLPA method, the typical genetic profile of individuals presenting a (δβ)° Spanish deletion. *Methods.* MLPA (Multiplex Ligation-dependent Probe Amplification) analysis of β globin cluster in 15 patients with the (δβ)° Spanish deletion in heterozygous state (detected previously by gap-PCR). This technique is based on the quantitative amplification and a subsequently fragment analysis of multiple probes hybridized across a region of interest. This method allows for a genetic profile showing the copy number of fragments of DNA that have been analyzed. A deletion is detected when a reduction of the amount of amplified product of several consecutive probes is observed in the genome. Here we used a commercial kit (SALSA MLPA kit P102-B1 HBB, MRC-Holland) that contains 28 probes designed to detect copy number changes in the beta cluster, from LCR to 10Kb downstream of β globin gene, spanning more than 80Kb. *Results.* All the patients showed an identical profile after MLPA analysis. The reduction of the amplified product was found in the same 12 probes, from the exon1 δ gene probe to the last probe located downstream of the β globin gene. These results are consistent with the previous works in which this deletion has been mapped. *Summary/conclussions.* Large deletions are usually difficult to be characterized by sequencing owing to the loss of entire genes. Uptime, the molecular characterization of this kind of deletions has required specific PCR based methods or laborious techniques like Southern Blot. In Spain the most frequently deletion associated to δβ-thalassemia is the (δβ)° Spanish. To know the cut-offs in the beta globin cluster allows the identification by PCR-Gap of this deletion, however, can not characterize other deletions. This study shows that MLPA is a rapid and reproducible technique for identification of this deletion. Because immigration changes probably will see a greater heterogeneity at the molecular level in the cases of (δβ)° thalassemia in our environment, so the study by MLPA will allow a correct characterization of the molecular basis of (δβ)° thalassemia.

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GROWTH AND DEVELOPMENT OF PAEDIATRIC PATIENTS WITH BETA-THALASSAEMIA TREATED WITH DEFERASIROX FOR UP TO 5 YEARS

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Background. Growth and development in β-thalassaemia patients may be adversely affected by iron-induced endocrine abnormalities or by iron chelator-induced toxicity, a well-recognised effect of intensive chelation with deferoxamine (DFO).[1] In study 107, a 1-year, Phase III trial, β-thalassaemia patients were randomized to receive the oral iron chelator deferasirox or DFO. Patients completing this core phase continued with deferasirox (deferasirox cohort) or switched to deferasirox (crossover cohort) during a 4-year extension. Approximately half the patients were paediatric, therefore it is of interest to assess the effects of long-term deferasirox on paediatric growth and development. *Aim.* To evaluate the effects of up to 5 years' deferasirox treatment on serum ferritin, growth and development of paediatric β-thalassaemia patients. *Methods.* β-thalassaemia patients with transfusional iron overload aged 2-<16 years who received deferasirox during the core and/or extension were included. Deferasirox dose was initially based on liver iron concentration at start of treatment; dose adjustments based on monthly serum ferritin and safety trends. Height, weight and sexual development were analyzed annually.

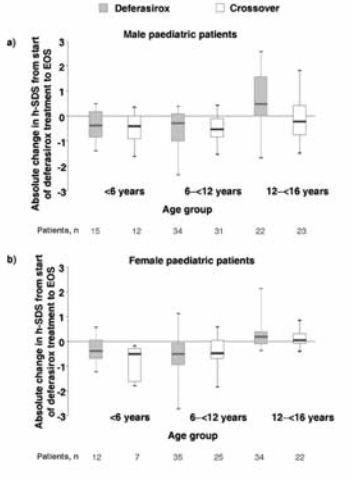

Figure. Absolute change in h-SDS start of deferasirox treatment to EOS for a) male and b) female paedriatric β-thalasseaemia patients receiving deferasirox or up to 5 years.

Results. 273 paediatric patients were included. In the deferasirox (n=153) and crossover (n=120) cohorts respectively, 28 and 19 patients were aged 2-<6 years, 69 and 56 were aged 6-<12 and 56 and 45 were aged 12-<16. Mean actual deferasirox dose in the deferasirox and crossover cohorts, respectively, was 25.2±6.1 and 23.2±4.2 mg/kg/day (2-<6 years), 22.7±6.4 and 23.7±5.5 mg/kg/day (6-<12 years) and 20.0±6.4 and 22.7±5.5 mg/kg/day (12-<16 years). Median serum ferritin decreased from 2409 to 1208 ng/mL after 5 years' deferasirox treatment (n=107, P<0.001) in the deferasirox cohort, and from 1922 to 1047 ng/mL after 4 years' deferasirox (n=83, P<0.001) in the crossover cohort. Overall, mean height standard deviation score (h-SDS) at start of deferasirox was -0.96±0.99; at end-of-study (EOS) was -1.17±1.03 (similar in males and females). Absolute change in h-SDS from start of deferasirox to EOS was not notably impaired in patients aged 2-<6 and 6-<12 years, whereas a positive change in patients aged 12-<16 years may indicate that deferasirox does not retard growth for both genders (Figure). Individual growth curves showed most patients were within normal percentile ranges and did not show growth impairment. In females aged 12-<16, the proportion of patients at Tanner Stage 5 for breast development increased from 8.8% to 51.5% and 18.1% to 72.7% from start of deferasirox to EOS in the deferasirox and crossover cohorts, respectively. In males none of the patients aged 12-<16 were at Tanner Stage 5 for testicular volume at start of deferasirox, whereas at EOS, 27.2% and 28.5% were at Stage 5 in the deferasirox and crossover cohorts, respectively. There were no clinically relevant differences in sexual development between deferasirox and crossover cohorts. *Conclusions.* Deferasirox treatment for up to 5 years was effective in decreasing serum ferritin down to maintenance levels of around 1000 ng/mL without an adverse effect on growth and sexual development in paediatric β-thalassaemia patients.

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COMPLICATIONS AND TREATMENT OF PATIENTS WITH β-THALASSEMIA IN FRANCE: RESULTS OF THE NATIONAL REGISTRY

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Background. β-thalassemia is rarely encountered in France (prevalence less than 1/100 000) where the disease is mainly diagnosed in subjects originated from Italy and Algeria. *Aim.* In order to improve clinical care, a national registry for Thalassemia has been developed since 2005. Epidemiological and clinical data are collected on living beta-thalassemia major (TM) or intermedia patients (TI), including those who received a HSC transplantation. *Results.* In January 2010, the French register included 437 patients, 303 TM, 125 TI and 9 not yet classified, 71% of them were born in France (74% for TM). The patients are spread all over the country in 65 centres, 46% of the patients being treated in 6 centres. Median age was 20 years (0-78). Iron overload related complication rates and serum ferritin levels are presented in Table 1 (univariate analysis). Rate of parenthood were not statistically different between TM and TI patients after age adjustment. Cardiac MRI was increasingly used over the last 4 years and was performed for 45% of TM patients aged more than 10. Deferoxamine was prescribed to 2/3 of the patients receiving chelation treatment during the years 2005-2006 while 70% received deferasirox over the years 2008-2009 and less than 10% a combination of DFP/DFO. A hematopoietic stem cell transplantation has been performed for 60 patients. *Conclusion.* French beta-thalassemia patients are treated all over the country in numerous centres. The global state of health appeared very similar to the reported data in Europe and North America, in particular concerning the median serum ferritin levels, the rates of iron overload related complications and the use of HSC transplantation This register is also a valuable tool for identifying clinical care aspects which should be improved in the future, especially the use of cardiac MRI and of chelation treatment with combined therapy.

Table 1. Iron related complications rates, parenthood and serum ferritin levels according to the type of thalassemia.

	Thalassemia Major	TM with successful HSC transplant	TM with Graft failure	Thalassemia Intermedia
Number of patients	245	48	10	123
Median age (yrs) ***	19	12,5	27	28
Diabetes **	17 (6.9%)	0	3 (30%)	5 (4.1%)
Cardiac failure*	26 (10.6%)	0	2 (20%)	5 (4.1%)
Hypogonadism **	76 (49.5%)	6 (28.6%)	7 (70%)	20 (21.5%)
Hypothyroidism****	24 (9.8%)	2 (4.2%)	3 (30%)	3 (2.4%)
%<or=-2SDs for height	19.5%	26%	30%	18%
Parenthood* 40F/22M	18.2% (25/137)	12.5% (2/16)	11.1% (1/9)	41% (34/83)
Median SF (ng/ml)***	**1278** (155-7890)	**748** (98-4989)	**1347** (413-7741)	**522** (13-5141)

***p<.001, **p<0,01; *p<0,05

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FATIGUE AND IMPAIRED QUALITY OF LIFE IN PATIENTS WITH PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH) IS ASSOCIATED WITH HEMOLYSIS, BUT NOT WITH ANEMIA

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Background. PNH is a progressively debilitating and life-threatening blood disease characterized by chronic complement-mediated intravascular hemolysis. Historically, physicians have tended to view and treat PNH as a disease of anemia. In particular, patients with PNH often suffer from severe fatigue and dyspnea (shown to be a measure of pulmonary hypertension), which contribute to impaired quality of life (QoL). Clinically, these measures of disease burden have historically been assessed by both the level of anemia and transfusion history. However, recent studies have demonstrated that primary patient symptoms, life-threatening complications and significantly shortened lifespan are also significantly associated with the degree of chronic hemolysis. *Aims/Methods.* The current investigation examines the impact of hemolysis and anemia on fatigue and dyspnea in patients with PNH.Twenty-nine Japanese PNH patients (14 men and 15 women; median patient age, 47 years; range 26-70 years) were treated with eculizumab for 12 weeks at 9 institutions in Japan participating in the AEGIS study. *Results.* Serum LDH decreased 87% from a median of 1,814 U/L at baseline to 244 U/L at 12 weeks of eculizumab treatment (P<0.001; normal range 103-223 U/L). The reduction in hemolysis led to significant improvement in dyspnea within 1 week of eculizumab treatment (P=0.02 from baseline) and the improvement was sustained throughout the study (P<0.001). Furthermore, 41% of patients reported a major improvement (10% or greater) in dyspnea (mean among general population = 11.8)1 with eculizumab treatment that was sustained through Week 12. Importantly, dyspnea is a primary characteristic of pulmonary hypertension, which has been found in other studies to occur in approximately 50% of PNH patients. Similar to dyspnea, improvements in fatigue (measured by the FACIT-Fatigue scale) were also observed at 1 and 2 weeks of eculizumab treatment (P=0.03; P<0.0001 respectively) (Table 1).

Table 1. Effects of eculizumab on fatigue, dyspnea and hemoglobin during first 4 week of treatment.

	Baseline	Change from Baseline Mean ± SE Median (P-value)			Overall Effect from Baseline *P*-value
		Week 1	Week 2	Week 4	
Fatigue-FACIT Score	38.5 ± 1.9 41.0	2.1 ± 1.1 2.0 (0.03)	4.2 ± 1.0 4.0 (<0.001)	4.9 ± 1.4 4.0 (<0.001)	<0.001 ‡
Fatigue – EORTC Score*	33.7 ± 1.1 33.3	-7.7 ± 2.3 -11.1 (<0.001)	-13.4 ± 2.5 -11.1 (<0.001)	-11.1 ± 2.9 -11.1 (<0.001)	<0.001 ‡
Dyspnea – EORTC Score*	37.9 ± 5.2 33.3	-11.5 ± 4.1 0.0 (0.02)	-13.8 ± 3.9 0.0 (<0.001)	-13.8 ± 4.5 0.0 (<0.001)	<0.001 ‡
Hemoglobin (g/dL)	7.9 ± 0.3 7.6	N/A	0.2 ± 0.2 0.0 (NS)	0.4 ± 0.2 0.0 (NS)	= 0.002 ‡

NS = not significant; *P*-value based on signed ranked test.
‡Overall was based on the average of least square means from Week 1 to Week 12.
* A negative change in EORTC score of fatigue and dyspnea indicates improvement.

Among the general population, the mean FACIT-Fatigue score is 43.6.2 By the end of the first week of treatment, 27.6% of treated patients experienced a clinically meaningful improvement in fatigue (at least a 4-point increase on the FACIT-Fatigue scale), while 51.7% improved at Week 2, and a sustained benefit was observed with 58.6% of patients reporting a clinically important improvement in fatigue at Week 12. The early improvement in fatigue measured by FACIT-Fatigue score was confirmed by a large median improvement in EORTC QLQ-C30 Fatigue score (among the general population mean = 24.1)1 of 11.1 points by Week 1 (P<0.0001). In contrast, hemoglobin did not significantly improve until week 8, and increased from 7.6 to 9.0 g/dL (P<0.001) by week 12 despite a concomitant reduction in transfusions. *Conclusions.* These data demonstrate that hemolysis, independent of anemia, drives the risks and burden of disease in PNH. Further, inhibition of terminal complement activation with eculizumab improves hemolysis, fatigue, dyspnea and other significant morbidities of disease, independent of anemia, in Japanese patients with PNH.

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FOUR NOVEL PKLR GENE MUTATIONS IN ASSOCIATION WITH PYRUVATE KINASE (PK) DEFICIENCY

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Background. Pyruvate Kinase (PK) deficiency is the most common enzyme abnormality in the erythrocyte glycolytic pathway causing chronic nonspherocytic haemolytic anaemia. The PK-R isozyme, exclusively expressed in erythrocytes, is transcribed from the PKLR gene (1q21). Clinical symptoms occur in patients homozygous or compound heterozygous for an abnormal allele and vary from severe haemolysis to a well compensated form of chronic anaemia. More than 190 mutations had been found associated with PK deficiency. *Aims.* To report the molecular characterization on 3 PK-deficient patients with chronic haemolytic anaemia and in 1 heterozygous individual, and to understand the genotype/phenotype correlation. *Methods.* Three patients with haemolytic anaemia, a 2-yr-old and a 3-yr-old Spanish girls and a 28-yr-old Portuguese woman, and another individual from Portugal, were referred to our Haematological unit for diagnostic purposes and molecular characterization. Haematological parameters and red blood cell PK enzyme activity were measured by standard *Methods.* After informed consent, genomic DNA was extracted from EDTA peripheral blood samples and PKLR gene was studied by PCR, SSCP and sequencing analysis. *Results.* The molecular analysis identified seven different PKLR mutations. All PK-deficient patients are compound heterozygotes for two different mutant alleles. Patient 1: Hb 92 g/L, reticulocytes 8.3% and PK activity 38% of normal, is a compound heterozygous for mutations IVS6(+2)T>G and c.1492C>T (p.Arg498Cys); Patient 2: Hb 74 g/L, reticulocytes 5.6%, is a compound heterozygous for mutations c.1463G>A (p.Arg488Gln) and c.721G>T (p.Glu241stop); Patient 3: Hb 127 g/L, reticulocytes 7% and PK activity 19% of normal, is a compound heterozygous for mutations c.815T>C (p.Leu272Pro) and c.1456C>T (p.Arg486Trp). Mutation c.491C>A (p.Thr164Asn) in heterozygous state was identified in an individual with normal haematological parameters and PK enzymatic activity 72% of normal. *Conclusions.* Four mutations are reported here for the first time: the splice site mutation IVS6(+2)T>G, and three missense mutations, predicting the substitution of well-conserved amino acids, p.Thr164Asn, p.Leu272Pro and p.Arg488Gln. Mutation IVS6(+2)T>G affects the invariant sequence GT located at the intron 6 donor splice site and most probably abolishes the normal splicing of the mRNA transcript. In patient 1 this mutation is associated in trans with the previously described missense mutation p.Arg498Cys (L-Cβ2-Cα4), resulting in a moderate clinical phenotype. The new mutation p.Arg488Gln affects a C domain (L-Cα3-Cβ2) residue. The side chain of Arg488 points to the PK subunit cleft between C and A domains and the Arg>Gln substitution affects the A/C interdomain interaction disturbing the enzyme structure and alosteric properties. The association in trans with the nonsense mutation p.Glu241stop, explains the severe chronic haemolytic anaemia in patient 2. Mutation p.Leu272Pro (Aα3) targets the hydrophobic core of A domain and was found in trans with the known substitution p.Arg486Trp in patient 3, causing a mild phenotype. The new substitution p.Thr164Asn, identified in a heterozygous individual, involves an amino acid change in PK B domain (Bβ1) near the cleft between A and B domains, where the active site is located. Until now, only 7 mutations had been described in the PK B domain.

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CHRONIC ORGAN DAMAGE IS NOT RELATED TO PREGNANCY COMPLICATIONS IN SICKLE CELL DISEASE

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Background. Patients with sickle cell disease (SCD) develop accumulating organ damage throughout their lives as result of chronic hemolytic anemia and ongoing vaso-occlusion. Chronic organ damage has been related to significant morbidity and increased mortality. Previous studies have shown significant increased fetal and maternal complications in patients with SCD. It is unclear whether the presence of chronic organ damage is related to pregnancy complications in these patients. *Aims.* To determine the relation between chronic organ damage and pregnancy complications in women with SCD. *Methods.* We performed a retrospective analysis of pregnancy complications in all women known with SCD (defined as HbSS, HbS-β^0, HbSC and HbSβ^+) in a teaching hospital in the Netherlands. Pregnancy complications consisted of: (pre)eclampsia, preterm birth, urinary tract infection, hypertension, still birth, perinatal mortality, maternal mortality, painful crisis and acute chest syndrome (ACS). In all patients organ damage (retinopathy, avascular osteonecrosis, microalbuminuria, pulmonary hypertension, chronic leg ulcers and cholelithiasis) and the history of sickle cell complications in the previous 5 years (pain rate >1 crises/year, ACS and stroke) was assessed. The patients were divided in a severe (HbSS/HbSβ^0) and a mild genotype group (HbSC/HbSβ^+). Chronic organ damage and the history of previous sickle cell-related complications were related to pregnancy complications, birth weight and laboratory tests. *Results.* All 96 female patients known with SCD in our hospital were systematically evaluated for organ damage and sickle cell related complications. Thirty-six patients had not been pregnant at time of evaluation, of 7 women medical information about their pregnancy was missing and 6 women were only known with an elective abortion. Forty-seven women with SCD (18 HbSS, 3 HbSβ0, 21 HbSC and 5 HbSβ^+) were evaluated for pregnancy complications. In the severe genotype group significantly more pregnancy complications were observed as compared to the milder group (18/21(86%) *vs.* 13/26(50%), P=0.011). Chronic organ damage or a history of severe sickle complications in the previous 5 years was present in 17/21(81%) of the patients with a severe genotype and 15/26 (58%) patients with a mild genotype. (P=0.13) No relation between pregnancy complications and the presence of organ damage or a history of sickle cell-related complications was found. Children of patients with the severe genotype had a significant lower birth weight as compared to the children of patients with a milder genotype (2606±675 *vs.* 3032±560 gram; P=0.027) which correlated with hemoglobin concentration (Hb) (r=0,432; P=0.05). When analyzed according to genotype, a weaker but no significant correlation between Hb and birth weight was observed (r=0.355, P=0.62 for HbSS/HbSβ0 and r=0.356, P=0.10 for HbSC/HbSβ^+). No correlation between Hb or LDH concentrations and pregnancy complications was found. *Conclusions.* The majority of patients with SCD had at least one pregnancy complication which was significantly more frequent observed in patients with a severe genotype. Birth weight was significantly lower in the severe genotype group which could partly be explained by a more severe anemia. However, no correlation between pregnancy complications and manifestations of chronic organ damage or a history of previous sickle cell-related complications was demonstrated.

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MYOCARDIAL IRON OVERLOAD ASSESSED BY MAGNETIC RESONANCE IMAGING (MRI)T2* IN MULTI-TRANSFUSED PATIENTS WITH THALASSEMIA AND ACQUIRED ANEMIAS

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Iron-induced cardiomyopathy is the commonest cause of death for patients suffering from thalassemic disease, as well a recurrent request in the case of multi-transfused patients with acquired anemias. MRI T2* is, at the moment, the gold standard for assessing myocardial iron. The aim of our study was to evaluate a population of patients of the southern Italy using MRI T2* and to find clinic and serologic parameters that might be correlated with this technique. Three subsets of patients were studied: 1) 95 with thalassemia major (TM), 30 years mean age (range 15-48); 2) 18 multi-transfused patients with thalassemia intermedia (TI), mean age 38 years (range 25-63); 3) 11 multi-transfused patients with acquired anemias (AA) (9 with myelodysplastic syndromes, 1 with aplastic anemia and 1 with primary myelofibrosis), mean age 75 years (range 66-85). In the latter group median number of packed red blood (PRBC) units was 77 (range 12-234); all were negative for HFE mutations and 7 out of 11 patients were on iron chelation. In the group 1), cardiac MRI T2* mean (±SD) values were 26±14msec; pathologic values (≤20 msec) were found in 36 (38%) patients. Three out of 95 patients had significantly impaired left ventricular ejection fraction (LVEF≤30%) assessed with echocardiography. Only 1 of these 3 patients had T2*≤20msec. Nine patients had LEVF within the range 30 to 50 %. Mean serum ferritin values were 1615±1834 ng/mL. We found a significant negative correlation only between serum ferritin and age (R=-0,33, P=0.001). We divided the patients according to their T2* values into subgroup 1a (T2*≤20msec, N=36), and 1b (T2*>20 msec, N=59). Comparing data between these subgroups, we found no difference either for age or chelation regimen; a difference, but not significantly, was found between serum ferritin values (557± 385, P=0.15). Besides, 17 (47%) patients in the subgroup 1a had serum ferritin values<1000 ng/mL *vs.* 38 (64%) patients in the subgroup 1b. In the group 2), cardiac MRI T2* mean values were 30±10 msec; pathologic value (16msec) was found in 1 patient. None had impaired ejection fraction. Mean serum ferritin values were 910±713 ng/mL. No correlation was found between cardiac T2* and ferritin. The mean difference in T2* values between group 1) and 2) was -4 msec (P=0.25). In the group 3) cardiac MRI T2* mean values were 34±10 msec; pathologic value (17.7msec) was found in 1 patient, who had received 234 PRBC units. Echocardiography results were in the normal range for the age. Mean serum ferritin values were 1936±1226 ng/mL. No correlation was found between cardiac T2* and ferritin. T2* values were negatively associated, but not significantly, with the number of PRBC transfused (R=-0,43, P=0,18). *Conclusion.* 1) in our experience 38% of TM patients had a myocardial iron overload assessed by MRI T2*, higher if compared to TI patients; 2) 47% of TM patients with pathologic T2* values had serum ferritin values<1000ng/mL, therefore serum ferritin measurement was a poor predictor of myocardial iron overload; 3) in patients with AA more than 200 PRBC units transfused are likely required to induce cardiac hemosiderosis.

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CARDIAC IRON OVERLOAD AND FUNCTION BY CMR IN DIFFERENT PHENOTYPIC GROUPS OF THALASSEMIA INTERMEDIA PATIENTS

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Background. Thalassemia intermedia (TI) indicates a clinical condition of intermediate gravity between the asymptomatic carrier and the transfusion-dependent form. The genetic bases of the TI are likewise variable. Cardiac involvement in TI patients seems to be more related to an high cardiac output state than to myocardial iron overload (MIO). The relationship between the presence of a precise phenotype and the cardiac impairment in TI has not been investigated. *Aims.* Our study aimed to detect if different phenotypes could be related to different levels end kind of cardiac involvement, evaluated by cardiovascular magnetic resonance (CMR). *Methods.* We performed a retrospective review of the CMR results and of clinical data about 52 TI patients (age 38±10 years, 54% females) enrolled in the Myocardial Iron Overload in Thalassemia (MIOT) project. In the MIOT network all CMR and thalassemia centers are linked by a web-based network, configured to collect patient anamnestic, clinical and diagnostic data. MIO was assessed using a multislice multiecho T2* approach. Cine sequences were obtained to quantify biventricular morphological and functional parameters. *Results.* Three groups of patients were identified: heterozygote (N=18), homozygote β^+ (N=19), homozygote β^0 (N=15). No significant

differences for sex and age were found among the groups. The global heart T2* value was significantly higher in the homozygote β° group compared to the homozygote β+ group (42±5.6 ms *vs.* 34±8 ms, P=0.006). The homozygote β° group showed significantly higher Left Atrial Area values than the heterozygote group (28±4 cm^2 *vs.* 22±5 cm^2, P=0.014). The homozygote β° group showed significantly higher left ventricular (LV) end-diastolic volume index (EDVI) than the heterozygote and homozygote β+ group (116±19 mL/m^2 *vs.* 94.7±21.7 mL/m^2 *vs.* 96±23 mL/m^2, P=0.019) and significantly higher LV mass index than the heterozygote group (70±15 g/m^2 *vs.* 57±14 g/m^2, P=0.032). The homozygote β° group showed significantly higher right ventricular (RV) EDVI and end-systolic volume index (ESVI) than the heterozygote group (113±24 mL/m^2 *vs.* 90±24 mL/m^2, P=0.031; 43±17 mL/m^2 *vs.* 29±10 mL/m^2, P=0.01). *Conclusions.* Heart remodelling related to a high cardiac output state cardiomyopathy was more pronounced in the homozygote β° TI patients. These data support the knowledge of the different phenotypes in the clinical and instrumental management of the TI patients.

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HELICOBACTER PYLORI RELATIONSHIP WITH IRON DEPLETION AND VITAMIN B12 DEFICIENCY

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Helicobacter Pylori (HP) infection has been associated with iron deficiency (ID) and poor response to oral iron. Moreover, some patients showed anti-parietal cell antibodies (PCA) and low vitamin B12, suggesting a relationship between ID, vitamin B12 deficiency and HP. *Objective.* To study in low vitamin B12 (LB12) patients the presence of ID and HP. *Methodsology.* Etiology of 198 patients with LB12 (serum vitamin B12 < 201 pmol/l) was evaluated using clinical findings, laboratory parameters and digestive studies. Clinical characteristics of patients with ID and LB12 were studied, including the presence of HP. Iron deficiency was diagnosed using iron variables (serum iron, total iron binding capacity, transferrin saturation, serum ferritin and soluble transferrin receptor). HP was confirmed by Breath test and/or gastric biopsy. *Results.* In 57 out of the 198 (28.8%) patients with LB12, ID was showed, including 42 (73.7%) with pernicious anemia (PA). Interestingly, macrocytosis or macrocytic anemia was demonstrated in 6 cases (10.5%) and microcytosis or microcytic anemia in 16 (29%). With regard to the 42 cases with PA, macrocytic and microcytic anemia was observed in 3 (7%) and 11 cases (26%), respectively. Anti-intrinsic factor antibodies (IFA) and PCA were demonstrated in 57% and 90% of patients, respectively. Serum gastrin > 60 U/l was increased in all cases. An increase of serum homocystetine (Hcy) (> 16.9 micromol/L) was showed in 20 out 23 cases in whom Hcy was determined. With regard to the 15 cases with LB12, ID and without PA, in 8 patients (53%) HP was positive, (4 were in treatment with omeprazole, 1 after gastroplastia and in 2 no etiology was found, but Hcy levels were normal, 8.6 and 9.8). In the 8 cases with HP, ID and LB12, serum B12 ranged from 121 to 158, Hcy was increased in 5 (62.5%) and Hcy decreased after treatment. IFA were negative in the 8 cases and PCA positive in 4 cases. Increased serum gastrin was also showed in 4 cases. Furthermore in 14 out of the 141 with LB12 without ID (10%) HP was positive. Interestingly, PCA were negative, but Hcy was increased in 12 out of 14 cases (from 17.5 to 35micromol/l) and Hcy normalized after treatment. An increase in serum methylmalonic acid (> 0,4 mmol/L) was showed in the 5 cases in which it was determined. Conclusion. In our experience, in 74% of cases with ID and LB12, this deficiency is caused by PA and few of them showed macrocytosis. In 26% of the remaining cases, HP is positive, most of them with a real vitamin B12 demonstrated by an elevated Hcy. Therefore, HP is probably the second cause of ID and LB12. Moreover, in some cases (approximately 10%) with vitamin B12 deficiency without ID, HP was also demonstrated. HP investigation should be carried out in patients with ID and LB12 and patients with LB12 without a clear cause of the LB12.

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HEREDITARY HYPERFERRITINEMIA-CATARACT SYNDROME (HHCS) PRESENTING WITH IRON DEFICIENCY ANEMIA AND THE IDENTIFICATION OF A NEW AND PATHOGENETIC RELEVANT HETEROZYGOUS MUTATION +24T>C (=HGVS C.-176T>C) IN THE IRON RESPONSIVE ELEMENT OF THE L FERRITIN GENE IN A SWISS FAMILY

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Hereditary hyperferritinemia-cataract syndrome (HHCS) is one of the differential diagnoses of hyperferritinemia (HF) with low or normal transferrin saturation (TS) but is usually not associated with anemia. We report a case of a 39-year old woman who was referred for an evaluation of a microcytic, hypochromic anemia with a hemoglobin of 102g/L (normal range 115-148 g/L), MCV 68fl (80-97fl), MCH 22 pg (27-34 pg) and an elevated ferritin of 383 μg/L (15-150 μg/L). Reticulocyte hemoglobin was 22.7pg (28-35pg), TS 9% (16-45%), soluble transferrin receptor 9.8 mg/L (1.9-4.4 mg/L) and ferritin index 3.79 (<3.2), which all was in favour of iron deficiency despite HF. HFE gene mutation studies showed a heterozygous mutation for H63D. Vitamin B12, vitamin B6, erythrocyte folate, copper, zinc and coeruloplasmin were all in the normal range, a hemoglobinopathy could be excluded. Bone marrow examination could exclude a myelodysplastic syndrome and revealed empty iron stores on Prussian blue staining, therefore the diagnosis of an iron deficiency anemia was made. To evaluate the cause of the HF with low TS further an ophthalmological examination was performed. In spite of absent visual impairment or glare there was evidence of bilateral cataract with scattered small snowflake-shaped opacities throughout the lens compatible with the diagnosis of HHCS. HHCS is an autosomal dominant disorder characterized by distinctive cataracts and HF in the absence of iron overload. Therefore sequencing studies were done to look for mutations in the iron responsive element (IRE) of the L ferritin gene. A heterozygous single point mutation for a +24T to C substitution in the IRE of the L ferritin gene (=HGVS c.-176T>C) was detected which has not been described before. To evaluate the pathogenetic relevance of this new mutation we performed family studies of parents and siblings. We could identify the father and one brother with HF, cataract and the heterozygous +24T>C mutation. Neither the mother nor the five other siblings had HF, cataract or that mutation. We therefore conclude that this here first described heterozygous +24T>C mutation in the IRE of the L ferritin gene causes HHCS. As our patient had a symptomatic iron deficiency anemia caused by hypermenorrhagia we substituted iron sucrose intravenously and applied 1200mg in total. Anemia and all laboratory parameters suggestive of iron deficiency normalized and ferritin was 506μg/l at the end of substitution. *Acknowledging.* HHCS is important as phlebotomies are contraindicated and cataract formation needs ophthalmological attention.

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AGE-RELATED COMPLICATIONS IN TREATMENT-NAÏVE PATIENTS WITH THALASSEMIA INTERMEDIA

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Background. Age-related complications have never been evaluated in patients with thalassemia intermedia (TI) partly because of the confounding effects of various treatment modalities used in any studied cohort. *Aims.* To evaluate the occurrence of several disease-related complications with advancing age in a large cohort of TI patients who never received any form of disease treatment. *Methods.* We re-evaluated data collected for the Thalassemia Intermedia Registry, a database of 584 TI patients currently registered at six comprehensive care centers in Lebanon, Italy, Iran, Egypt, United Arab Emirates, and Oman. Institutional review boards (IRBs) at each center approved the study protocol. Patients who never received any treatment intervention (splenectomy, transfusion therapy, iron chelation therapy, fetal hemoglobin inducing agents) were identified and included in this study (n=120). Retrieved data included demographics (age and gender); identified mutation; mean hemoglobin (Hb) and steady-state serum ferritin (SF) levels of three con-

secutive measurements within the year corresponding to patient age; and presence of complications (extramedullary hematopoiesis [EMH], leg ulcers, thrombosis, pulmonary hypertension [PHT], heart failure [HF], cholelithiasis, abnormal liver function [ALF], diabetes mellitus [DM], hypothyroidism, osteoporosis, and hypogonadism). Patients were divided into four quartiles (n=30 each) according to their age: < 10, 11-20, 20-32, and > 32 years. *Results.* The mean age of the patients was 21.4±13.4 years (range: 2-56 years). The male to female ration was 61:59. Homozygosity for IVS-I-6 (T -> C) was the most common mutation (87.5%), followed by IVS-I-5 (G -> C) (8.3%), IVS-II-1 (G -> A) (2.5%) and Codon 39 (C -> T) (1.7%). There was no statistically significant difference between age quartiles in the proportion of patients with co-inheritance of alpha thalassemia or determinants associated with increased gamma-chain production. The mean Hb and SF levels of the whole study group were 7.7±1.6 g/dL (range: 4.1-11 g/dL) and 610.7±515.1 ng/mL (range: 16.7-2520 ng/mL), respectively. There was a statistically significant negative correlation between age and Hb level (r = -0.679, P<0.001) and a statistically significant positive correlation between age and SF (r = 0.653, P<0.001). Between age quartiles, there was a statistically significant difference in the rate of EMH (P=0.007), leg ulcers (P=0.034), and DM (P=0.030), but not thrombosis (P=0.082), PHT (P=0.159), HF (P=0.664), cholelithiasis (P=0.103), ALF (P=0.536), hypothyroidism (P=0.069), osteoporosis (P=0.124), or hypogonadisim (P=0.055). However, with advancing age, there was a statistically significant trend towards a higher rate of EMH (P=0.001), leg ulcers (P=0.004), PHT (P=0.010), thrombosis (P=0.030), hypothyroidism (P=0.039), and osteoporosis (P=0.018). *Summary/Conclusions.* Despite having a milder form of the disease at initial presentation and diagnosis, TI patients are still at risk of acquiring several serious complications as they become adults.

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COMPILATION OF ALPHA-CHAIN HEMOGLOBINOPATHIES THAT OCCUR WITH MICROCYTOSIS

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Background. The molecular basis of most _-thalassemias are deletions in some of _ globin genes. However, about 5-10% of the cases are due to point mutations in these genes. The majority of these mutations are unique to the individual family, nevertheless some alterations such as mutation in the initiation codon and 5 bp deletion in the donor site of first intron know as Nco and Hph mutations respectively, are more frequent in some populations. *Aims.* In this work we show point mutation cases in α-globin genes diagnosed in our hospital during 2009, except to Nco and Hph mutations which have been ruled out. *Methods.* In 2009, 186 samples collected by the Hematology department of the Hospital Clinico San Carlos in Madrid were studied to show microcytosis with normal HbA2 and HbF, and without iron deficiency. Which 170 was due to deletions (91.4%) and 8 to the mutation Hph (4.3%). The geographical origin of patients covers all the spanish territory. For the molecular study was necessary the genomic DNA extraction from peripheral blood leukocytes, employing a Bio-Robot EZ1. α gene deletions, Nco and Hph mutations were ruled out by α-thalassemia StripAssay. The remaining 8 samples were studied by automated DNA sequencing with BigDye v1.1, specific for α2 and α1 genes. *Results.* This study found the following variants of hemoglobin, all in heterozygote condition: Hb Agrinio [CD29 CTG->CCG (Leu->Pro)α2] found in 2 different families; CD 23 [CD23 GAG->TAG (Glu->AMB)α2]; Hb Groene Hart or Bernalda [CD119 CCT->TCT (Pro->Ser)α1] in 2 families; Hb Plasencia [CD125 CTG->CGG (Leu->Arg)α2] found in 2 different families and Hb Utrecht [CD129 CTG->CCG (Leu->Pro)α2]. *Conclusions.* In recent years the migratory movements have had a great relevance in the genetic structure of populations, significantly changing the frequency of molecular alterations in the globina genes. The identification in 8 different families of 5 point variants different from Nco and Hph mutations, reveals the molecular heterogeneity of cases of non-deletional α thalassemia in our average. This heterogeneity can have important implications in their phenotypic expression interacting with other more common deletion and non-deletional forms of α thalassemia, which are increasing in our average by the migratory changes. The integration of different molecular techniques, especially the automatic DNA sequencing, has a great importance for the clinical diagnosis of thalassemic syndromes mainly in non-deletional α thalassemia rare cases. The diagnosis of less frequent variants of this disease is essential to be applied to genetic counselling and prenatal diagnosis, and thus decrease sanitary and social costs that the treatment of these pathologies leads.

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THE EFFECTIVENESS OF LIPOSOMAL AMPHOTERICIN IN THE TREATMENT OF INVASIVE FUNGAL INFECTIONS IS NOT AFFECTED BY PRIOR AZOLE ADMINISTRATION: THE AMBI-PROF STUDY

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Background. It is a matter of debate whether mold-active azole prophylaxis may reduce the effectiveness of Liposomal Amphotericin (L-AmB) due to the potential antagonism in antifungal mechanisms. Recent data suggested that sequential azole and L-AmB did not reduce the efficacy of L-AmB (Cornely O, JAC 2010). *Aim.* To determine the non-inferiority of prior azole administration in the efficacy of treatment of Invasive Fungal Infections (IFI) with L-AmB in hematologic and allogeneic HSCT patients. *Methods.* We retrospectively studied patients who met the EORTC/MSG criteria for IFI and received treatment with L-AmB. Eligible patients were distributed in two arms according to: (A) mold-active azole exposure prior to L-AmB, and (B) fluconazole or no prior azole. Patients were stratified according to the type of IFI and evaluated for disease related risk factors and comorbidities at the time of L-AmB therapy. The primary endpoints were favorable response, defined as complete or partial response, and survival at the end of antifungal therapy, at 4 and after 12 weeks. *Results.* From Feb/2008 to Sep/2009, 182 consecutive patients were recruited from 26 institutions. The median age was 45 years (range 1-78). Most patients undergoing chemotherapy had acute leukemia (AL) or myelodysplasia (MDS) (129; 70.0%). Baseline disease was treated for induction, post-remission, or refractory/relapse status in 23.6%, 45.0% and 31.4%, respectively. A 40.1% of patients had received an allogeneic HSCT. A severe comorbidity and prior IFI were present in 20.3% and 14.8%, respectively. Arm A included 100 patients with prior itraconazole 39%, voriconazole 35% and posaconazole 26%. Arm B included 82 patients with fluconazole 49% or no azole 51%. Patients characteristics were not different in both arms, except for more cases of AL or MDS (P=0.002) and prolonged neutropenia in arm A (P=0.021), and more use of high dose steroids in arm B (P=0.01). The rates of possible, probable and proved IFI were 52.7%, 28.6% and 18.7%, respectively. Aspergillosis was the proven IFI in 28 of 35 cases. L-AmB was given at 3 mg/kg/d a median of 18±17 days in arm A and 15±13 in arm B. Up to 58% of patients in arm A, and 75% in arm B continued therapy after L-AmB with a different antifungal. Time to achieve a favorable response was 21 days in arm A and 27 days in arm B (P=0.252). The favorable response rate to L-AmB was 75.0% and 74.4% in both groups, with no differences in the responses at the end of treatment, at 4 weeks or at 12 weeks. The response rates for possible and probable or proven IFI were similar in both groups but survival was reduced in arm A (Table 1).

Table 1.

Table 1 **L-AmB treatment outcome**	**Group A** prior azole N=100	**Group B** no azole N=82	**Total** N=182	p value*
	n (%)	n (%)	n (%)	
Favorable response[1]	75 (75.0)	61 (74.4)	136 (75.0)	*ns*
Possible IFI	44 (83.0)	32 (74.4)	76 (79.2)	*ns*
Proven or Probable IFI	31 (66.0)	29 (74.4)	60 (69.8)	*ns*
End of treatment				
Favorable Response	73 (73.0)	55 (67.1)	128 (70.3)	*ns*
Survival	83 (83.0)	70 (85.4)	153 (84.1)	*ns*
At 4 weeks				
Favorable Response	57 (57.0)	50 (61.0)	107 (58.8)	*ns*
Survival	62 (62.2)	64 (78.0)	126 (69.2)	*0.020*
At 12 weeks				
Favorable Response	41 (41.0)	41 (50.0)	82 (45.0)	*ns*
Survival	42 (42.0)	49 (59.8)	91 (50.0)	*0.017*

*Group A vs B
[1] Number of complete or partial responses obtained at any time throughout the 12 weeks of the study

The Cox-odds ratio for death was 0.013 with a favorable response, 3.129 for arm A, 3.479 for high risk disease and 5.389 for refractory disease the time of L-AmB treatment (P<0.05). *Conclusions.* Prior exposure to

mold-active azoles does not seem to affect the effectiveness of L-AmB for the treatment of IFI in this high risk patient population, further indicating that a reduction in the response rate is not expected when given sequentially.

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SIGNIFICANT AND SAFE REDUCTION OF TRANSFUSION REQUIREMENTS BY CHANGING FROM DOUBLE- TO SINGLE-UNIT RED BLOOD CELL TRANSFUSION STRATEGY

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Background. Traditionally, single-unit red blood cell (RBC) transfusions are believed to be insufficient for correction of hyporegenerative anemias during intensive chemotherapies and stem cell transplantation. Although suggested by some guidelines, single-unit transfusion has neither been incorporated into modern transfusion medicine nor studied appropriately. *Aims.* Based on theoretical calculations of reducing the transfusion requirements, we have changed our transfusion policy in hospitalized patients from double- to single-unit transfusions by dispending only one RBC unit at the time from the in-hospital blood bank. Operation areas and intensive care units were excluded from this change. *Methods.* Here, we retrospectively evaluated the impact of the single-unit transfusion policy by comparing two patient cohorts before and after the change of the transfusion policy. *Results.* During the study period 139 patients received 274 therapy cycles and 1553 RBC transfusions. 135 (49%) cycles were administered during the double-unit and 139 (51%) during the single-unit period. During the double-unit period, in 78% of the transfusions two units were administered simultaneously, while only one RBC unit was given in 87% of the transfusions during the single-unit period. The mean units per transfusion during the two periods were 1.76 (range 0-4) and 1.12 (0-4), respectively (P<0.001). RBC transfusions were given at hemoglobin levels of 64g/L and 61g/L in the double- and single-unit period with a median increase of 14g/L and 6g/L with two and one RBC unit. The hemoglobin value at the start of the therapy did not differ between the two groups (89 *vs.* 90 g/L) while patients during single-unit period had slightly lower hemoglobin levels at the end of the therapy (74 *vs.* 78.5 g/L, P=0.001). The median time of aplasia was similar among the two groups (17 *vs.* 18 days, P=0.304). Single-unit transfusion strategy resulted in a 27% reduction of RBC requirements per therapy cycle (mean 9.6 *vs.* 7.0, P=0.002). Likewise, the mean number of RBC units per day of aplasia was significantly reduced (0.66 *vs.* 0.40 units per aplasia day, P=0.028). In a linear regression model, the change from a double-unit to a single-unit transfusion policy remained independently associated with a significant reduction of 2.5 units per cycle (P=0.002) when controlled for the duration of aplasia and the therapy regimen. Importantly, the overall survival of the single unit-cohort with a median follow-up of 118 (9-872) days was not different compared to cohort with the double-unit transfusion strategy (P=0.934 by log-rank test). *Conclusions.* The adherence to a single unit transfusion policy is excellent and the change from double- to single-unit transfusion strategy has the potential of saving approximately 25% of RBC units. The single-unit transfusion strategy proves to be safe and cost-effective and reduces the risks associated with allogeneic blood transfusions.

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ROUTINE ANTENATAL ANTI-D PROPHYLAXIS - IS THE PROTECTION ADEQUATE?

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Background. The use of Routine Antenatal Anti-D Prophylaxis (RAADP) was recommended by the National Institute for Clinical Excellence (NICE) in the U.K. in 2002 in order to prevent sensitisation of RhD negative women during the last trimester of the pregnancy. *Aims.* Prophylactic anti-D (IgD) given during a pregnancy can be detected in current indirect antiglobulin tests (IAT), using this as a measure of the persistence of prophylactic anti-D, this study set out to determine whether there was a correlation between IgD detectable at delivery and the RhD status of the foetus and /or the duration of the pregnancy post the standard 28 week dose of the RAADP. The study also investigated whether there was a difference in the detection of IgD at delivery if given in a two dose regime (2 doses of 500i.u at 28 and 34 weeks) or a one dose regime (one dose of 1500i.u. given at 28 weeks). *Methods.* All antibody screening IAT tests were undertaken using fully automated Diamed gel technology using LISS/Coombs IAT screening cards and Diamed 3 cell antibody screening cells. The results from 407 women were included in the two dose regime study, and 157 in the one dose regime study. All of these women had traceability data confirming the administration of RAADP. *Results.* Of the 407 women on the two dose regime 160 (39%) had no detectable IgD at delivery. The number of women delivering RhD positive and negative infants and the relationship with detectable IgD at delivery was analysed using the Fishers two-tailed test (with the level of statistical significance set at a P<0.05). This gave a P value of 0.59, showing that there is no correlation between the RhD status of the foetus and detectable IgD at delivery. The duration of the pregnancy and whether IgD was detectable at delivery was also analysed, giving a P<0.0001. This demonstrates that there is a strong correlation present between the time lapse from the 28 week dose to delivery and detectable IgD at delivery.Of the 157 women on the one dose regime 123 (78%) had no detectable IgD at delivery. Analysis of the relationship between IgD detectable at delivery and the RhD status of the infant gave a P value of 0.32 which again suggests that there is no correlation between these two variables. The relationship between the duration of the pregnancy and IgD detectable at delivery gave a P value of <0.0001 which confirms a strong correlation between whether IgD is detectable at delivery and the duration of the pregnancy post the 28 week dose. *Summary.* Our data show that neither the two dose regime nor the one dose regime appear to provide adequate cover at delivery for a large percentage of pregnant women, especially if those pregnancies go beyond 12 weeks from the administration of the primary dose.

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RED BLOOD CELL ALLOIMMUNIZATION AND FEMALE SEX PREDICT THE PRESENCE OF HLA ANTIBODIES IN PATIENTS UNDERGOING LIVER TRANSPLANT

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Background. Refractoriness to platelet transfusion due to HLA antibodies poses a serious risk to patients who require platelet support during surgery. Since routine screening for HLA antibodies would pose a tremendous burden on presurgical work-ups, a stratification of patients according to the risk of having HLA antibodies is warranted. *Aims.* To determine the prevalence of red blood cell (RBC) and HLA immunization in a large number of patients submitted to liver transplant and, to estimate the accuracy of RBC alloimmunization and other patient features to predict the coexistence of HLA antibodies. *Material and Methods.* Every patient considered for liver transplant in our hospital is routinely tested for the presence of RBC and HLA antibodies in the pretransplant workup. RBC antibodies are determined by a LISS-based antiglobulin test against commercial panels of reagent RBCs. The screening for HLA antibodies is done by lymphocytotoxicity against a panel of 20 selected cells. For the purpose of this study, the clinical and laboratory records of patients submitted to liver transplant from 1988 to 2008 (n=1491) were reviewed. Patients younger than 16 years and those not tested for HLA antibodies were excluded from the analysis. Predictive variables were selected through binary logistic regression and their accuracy to prognosticate HLA alloimmunization was calculated by contingent table Methods. *Results.* 1351 patients were included in this study. Median (range) age at the time of liver transplant was 53 (16-70) years and 876 (65%) were males. HCV and alcohol related cirrhosis constituted the 65% of the diagnosis. Seventy (5.2%) patients had RBC alloantibodies. The most common individual specificities were D (23 cases), E (21), C (19), Kell (19), c (6), e (6), Fya (5), and Jka (5). Twenty-two patients presented with two or more RBC alloantibodies simultaneously. HLA antibodies were detected in 71 (5.3%) patients. They include five cases of anti-HLA-A2 and one case each of anti-HLA-A10 and anti-HLA-A12. The remaining 64 cases had no definite specificity. The panel reactivity activity was 100% in 29 cases and ranged from 50-99% in 42 cases. Female sex and RBC alloimmunization were the only features independently associated with HLA aloimmunization. The risk for having HLA antibodies increased from male patients without RBC alloimmunization (n= 842; 1.5% with HLA antibodies) to female patients with RBC alloimmunization (n= 36, 36.2% with HLA antibodies), being of 9.7% in the remaining 473 patients. In females, the positive and negative predictive values of RBC alloimmunization to prognosticate HLA alloimmunization were 36% and 90%, respectively. In male patients, such values were 12% and 99%, respectively. *Conclusions.* RBC alloimmunized female patients submitted to elective surgical procedures that may require platelet support should be preoper-

atively evaluated for HLA antibodies. Such evaluation is unnecessary in male patients without detectable RBC antibodies. Decision-making in the remaining patients must be individualized.

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INCIDENCE AND CARACTERISTS OF SERIOUS ADVERSE EFECTS RELATED TO TRANSFUSION

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Background. Blood component transfusion can lead to undesirable side effects which range from mild complications to life-threatening reactions. Data from the incidence and characteristics of serious adverse events related to transfusion are required in order to install preventive strategies to increase the transfusion safety. The aim of this study is to analyze the incidence and characteristics of serious adverse events after blood component transfusion. *Study design and methods.* Between January 1st 2000 and December 31th 2009 all side effects related to transfusion were recorded as a part of our hemovigilance program. For all cases, adverse events were analyzed for its direct relation with transfusion and its severity, including the treatment response whenever applicable. For the purpose of this study only side effects with long term-morbidity (severity 2) or direct life-threatening (severity 3) or death (severity 4) that could be explained for the transfusion procedure (grade 2 association) or in which the transfusion was the certain cause (grade 3 association), were included in the analysis. All hemolytic, immune and infectious complications were registered for each of the blood component transfused. *Results.* During the study period a total of 359.671 units of red blood cells (234.805), platelets (70.431) and fresh frozen plasma (54.435) were transfused. The incidence of serious adverse events according to the previously defined criteria was 0.01%, which means 40 SAEs. Three additional SAEs were reported with the use of other blood or cell components. The incidence of SAEs was clearly higher for platelet transfusion than red blood cell transfusion (P<0.0001) and also higher for fresh frozen plasma (P=.04). The majority of SAEs were allergic/anaphylactic or febrile reactions (26 of 40) followed by hemolytic reactions (5 cases) and TRALI (4 cases). The majority of SAEs (83.7%) were of grade 2 severity and the patients survived without problems after treatment. These SAEs include all the hemolytic reactions, errors in blood component administration, febrile and hypotensive reactions, allergic or anaphylactic reactions, 2 cases of TRALI and 1 case of bacterial contamination. Three cases were grade 3 severity with serious consequences for the patients (one chronic respiratory failure after TRALY and two cases of human immunodeficiency virus infection). Four patients died: two of them due to TRALY after platelets transfusions, one due to septic shock and one allogeneic stem cell transplant recipient due to a CMV infection. *Conclusions.* The safety of blood components transfusion has been increased during last years. The incidence of SAEs is low (0.01%) with a mortality rate of 0.001%. However, SAEs can result in severe consequences for the patients and efforts should be made to reach a six sigma quality confidence level. Control of platelet transfusion indications seems to be an urgent measure to reduce transfusion related mortality.

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ROUTINE AUTOLOGOUS BLOOD COLLECTION AND SUBSEQUENT TRANSFUSION FOR DONORS OF BONE MARROW (BM) IS UNNECESSARY

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Background. Australian Bone Marrow Donor Registry and World Marrow Donor Association guidelines stipulate harvest centres should collect ≥1 autologous red cell units from allogeneic donors for transfusion during or after a marrow harvest. However, the necessity for routine autologous collection has not been established. This practice increases cost and inconvenience and does not eliminate the most significant transfusion associated risks, including bacterial contamination and administrative error. *Aim.* To perform a single centre retrospective analysis of the utility of autologous blood collection pre-allogeneic BM donation and review transfusion practices of stored autologous units. *Methods.* Fifty-nine consecutive BM donors presenting between January 2004 and December 2008 were identified. Sibling and volunteer unrelated donors were included. Haemoglobin (Hb) measurements pre-autologous blood collection, pre-harvest and post-harvest (prior to transfusion), where available, were retrospectively analysed. Transfusion of autologous units was audited. *Results.* The donors comprised 34 males (mean age 40yrs-range:16-52) and 25 females (mean age 39yrs-range:24-66). Forty-seven donors had autologous blood collected (33:1unit and 14:2units). The mean Hb pre-autologous donation for males (n=27) was 156 g/L (range:139-179 g/L) and for females (n=20) was 137g/L (range:116-157g/L). The mean reduction in Hb post-autologous collection was 12 g/L (-31 g/L to -1 g/L) and 7g/L(-30 g/L to +4g/L) for males (n=23) and females (n=17) respectively, despite routine iron supplementation. The mean BM harvest volume was 1068 mL (range:200-1725ml). The mean post-harvest Hb pre-transfusion in males (n=19) was 122g/L (range:92-151 g/L) with a mean drop of 23g/L (range:5-43 g/L), for females (n=12) the respective results were 108g/L (range:84-145 g/L) and 26 g/L (range:10-49 g/L). No donor who had a post-harvest Hb measured met National Health and Medical Research Council minimum criteria for transfusion (Hb<70 g/L). Twenty-seven of the 47 donors who had autologous blood collected, were transfused, although in 56% their Hb pre-transfusion was not checked. There was no significant difference in post-harvest Hbs of transfused (pre-transfusion 115g/L range:84-151g/L) *vs.* non-transfused donors (118 g/L range:88-145 g/L). *Conclusion.* Routine autologous blood collection prior to BM harvest leads to a drop in Hb pre-harvest, wasting of blood and unnecessary transfusions. Post-harvest Hb did not decrease to levels considered detrimental to healthy persons in any donor. We conclude routine autologous blood collection from healthy BM donors is unnecessary.

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DAILY REVIEW FOR UNDERTRANSFUSION

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Background. Most published reviews and audits of blood and blood-component transfusion have focused on the issue of overtransfusion and the inappropriate use of red cell components.[1] Efforts to curb unnecessary transfusion may result in a trend towards undertransfusion of patients.[2,3] There is little published information addressing this matter or the magnitude of its practice.[4] We herein report a study of the daily review of transfusion in the University Hospital of Salamanca, a 912-bed tertiary-care facility. *Aims.* The main objective is to detect the inappropriate use of red cell components due to undertransfusion, and, where this is found, secondarily, to determine whether the undertransfusion confers an increased risk of a negative outcome on the patients.[5] *Study design and methods.* Undertransfusion was evaluated by examining daily transfusion records of 3,201 patients for a 12-month period. Patients transfused in the previous 24 h, whose pretransfusional haemoglobin level was <7.5 g/dL were reviewed and evaluated for clinical undertransfusion criteria. *Results.* Over the 12-month period (February 2009 - January 2010) a total of 15,294 red blood concentrates were transfused by the University Hospital of Salamanca in 3,201 patients, 396 of whom were reviewed. The median age was 72 years; 75% of the population were over 64 years old, and 50% of the patients were older than 78 years of age. 36.4% (144) patients met the clinical criteria for undertransfusion. 41% (91) of these patients attended the Accident & Emergency Unit; their median age was 84 years. One of the undertransfused patients presented with an acute myocardial ischemia due to a sustained low haemoglobin level following orthopaedic surgery, and required admission to the Coronary Unit. After the review it was found that low haemoglobin levels (<7.5 g/dL) and symptomatic anaemia had been sustained during the previous week. *Summary/Conclusions.* Undertransfusion may occur in older patients (80+ years old), most of whom arrive in the Accident & Emergency Unit from an institution. Death or morbidity related to low or delayed transfusion are poorly documented, probably because there is no detection network and no methods dedicated to the detection of low or delayed transfusion. The population at greatest risk of undertransfusion are old people who live in institutions, where signs and symptoms may not be detected in time and where provision of care and a good diet is lacking.

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HIGH ACTIVATION STATE OF APHERESIS PLATELETS AFTER LOW VOLUME CENTRIFUGATION DURING MULTICOMPONENT COLLECTION

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Oncologic and hematologic patients treated by chemotherapy and possibly stem cell transplantation are usually poly-transfused and therefore exposed to multiple donor-derived blood products. Multicomponent collection (MCC) in apheresis enables the production of various standardized blood components during one donation session and may help to reduce the donor exposure to poly-transfused patients. In this paired prospective study we prepared platelet concentrates (PCs) by MCC using two different cell separators to compare metabolic, functional and activation parameters of platelets (PLTs) immediately after apheresis and during storage. Twenty-four donors underwent MCC donation on two different cell separators (Fenwal Amicus® and Caridian BCT Trima Accel®) within an interval of at least two months where one double dose of PLTs and one unit of packed red blood cells were collected. These two devices differ in the mode of PLT collection as in the Amicus® separator centrifuged PLTs remain highly concentrated as so-called "dry PLTs"within the collection chamber and are manually re-suspended in plasma at the end of apheresis. In contrast, in the Trima Accel® separator, PLT rich plasma is continuously collected outside the cell separator during centrifugation. On days 0, 2 and 7, PCs were tested for metabolic parameters and PLT function by aggregometry, rotation thrombelastometry and hypotonic shock response. PLT activation was analyzed by flow cytometry. Until day 7, metabolic parameters were well maintained in both groups. PLTs collected by the Amicus® device were significantly more activated as evidenced by higher CD62P and CD63 expression as compared to Trima PCs. This was observed in parallel to impaired *in vitro* PLT function revealed by aggregometry, hypotonic shock response and also partly rotation thrombelastometry. In multicomponent apheresis, standardized PC collection is effective and well tolerated. The higher activation of PLTs derived from the Amicus® separator may be due to the distinct modality of PLT collection. However, the causes for impaired PLT function and possible consequences for the clinical outcome have to be evaluated in further studies.

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BLOOD AND MARROW-DERIVED PROGENITORS ARE SUSCEPTIBLE TO MAPK P38-DEPENDENT FOAM CELL INDUCTION

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Clinical trials for therapeutic angiogenesis use blood- or marrow-derived transplants containing endothelial progenitor cells (EPCs), myelomonocytic cells and mesenchymal stromal cells (MSCs) to support vascular regeneration. Safety concerns have emerged since all three cell types can also contribute to atherosclerosis. We therefore asked whether human monocytes, EPCs or MSCs do in fact accumulate lipid droplets (LDs) and form foam cells *in vitro* as a surrogate marker for potential pro-atherogenic side effects of therapeutic angiogenesis. LD accumulation was quantified by flow cytometry, confocal laser scanning microscopy and cholesterol measurement in each of the cell types following exposure to low density lipoprotein *in vitro*. The impact of an initial three-day pro-angiogenic culture on subsequent foam cell formation was studied to mimic a relevant setting already used in clinical trials. The phosphorylation state of intracellular signalling molecules in response to pro-angiogenic stimulation was determined to delineate the operative mechanisms and to establish a basis for interventional strategies. Foam cells developed from monocytes but not from EPCs or MSCs after pro-angiogenic induction. The mitogen-activated protein kinase (MAPK) p38 phosphorylation related to foam cell development and stress-induced stimuli was enhanced in monocytes after pro-angiogenic stimulation. MAPK p38 inhibition almost abrogated intracellular LD accumulation *in vitro*. These data raise serious concerns that cellular therapy with hematopoietic cell preparations containing monocytes may be counterproductive or even aggravate atherogenesis in patients with cardiovascular diseases. We therefore support the argumentation that the role of transplanted cells in the various aspects of vascular homeostasis, regeneration and therapeutic angiogenesis must be re-examined prior to further clinical trials.

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INFORMED CONSENT AND PATIENT UNDERSTANDING OF BLOOD TRANSFUSION

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Background. Patient autonomy and informed consent underpin medical ethics and clinical governance. For consent to be valid, the person giving consent must be competent, acting voluntarily and be provided with sufficient information to make an informed decision. The minimum elements of informed consent to treatment are: 1) a description of the risks and benefits, the expected outcome without the treatment and available alternatives; 2) the opportunity to ask questions; and 3) the right to accept or refuse treatment. Obtaining separate informed consent for blood component transfusion is mandatory in some countries. Although there is widespread agreement that patients ought to be informed about risks and benefits of transfusion, studies in the United Kingdom suggest this does not happen as routinely as it should and the patient perspective is lacking in current literature. *Aims.* This study aimed to assess patient recall of the consent process, the information conveyed (verbally or by Blood Transfusion Patient Information Leaflet) and ease in understanding discussions concerning blood transfusion. *Methods.* A previously validated questionnaire was used as a framework in assessment of patient recall. All 342 adult patients for whom blood was cross-matched between 1st March and 30th April 2009 were sent postal questionnaires, whether transfused or not. *Results.* One hundred and sixty-four of the 342 questionnaires were returned. Overall, 59.1% of patients said someone had explained they might need a blood transfusion; of those 86.7% felt the reason why they might need a blood transfusion had been explained. Only 58.8% of patients felt they had been informed of what blood transfusion involves, whereas 67.0% said they had been informed of benefits of blood transfusion. When asked if they felt the information they received was explained in a way they could understand, 51.5% of patients agreed, but only 26.8% were aware of the Blood Transfusion Patient Information Leaflet. Of those who received the leaflet, all said they read it and had no questions. A particularly low compliance with standards was related to risks of blood transfusion, with just 27.8% of all patients feeling they had been informed of risks. Despite this, 61.9% of patients said they were satisfied overall with the information they received about blood transfusion. *Conclusions.* Although the level of recall and patient satisfaction exceeds that of many studies, there is room for improvement. Given time constraints in busy District General Hospitals such as The Great Western, a Blood Transfusion Patient Information Leaflet could prove invaluable in increasing the level of information available to patients, with minimal impact on health care professionals' time. These leaflets are available, free of charge, from the United Kingdom National Blood Service website and could be distributed relatively easily. These are currently being introduced at each bedside, in pre-operative information packs and in Outpatient Clinics, with re-audit of compliance with standards planned in six months.

Stem cell transplantation 2

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ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION WITH REDUCED-INTENSITY CONDITIONING IN PATIENTS WITH REFRACTORY AND RELAPSING MULTIPLE MYELOMA: LONG-TERM FOLLOW-UP

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Background. Allogeneic stem cell transplantation (SCT) with myeloablative conditioning is potentially curative therapy for multiple myeloma (MM) but is associated with excessively high rates of non-relapse mortality (NRM). Reduced-intensity conditioning (RIC) allows reduction of NRM compared with myeloablative conditioning but relapse rate is increased. The role and timing of allogeneic SCT during the disease course are controversial. There is only limited data on the long-term outcome of RIC in the relapsing/ refractory setting. *Methods.* We retrospective analyzed SCT outcomes in 50 patients given RIC for relapsing/ refractory MM between the years 2000-2004. Patients given an auto/allo tandem transplant were not included in this analysis. RIC consisted of fludarabine and melphalan (100-140 mg/m^2). Disease response was assessed at day +100. *Results.* The median age was 53 years (32-64). Donors were HLA-matched related (n=27) or unrelated (n=23) donors. This was a relatively heavily pretreated patient group, a median of 3 years from diagnosis (0.5-14 years). Forty-seven patients failed one (n=31) or two (n=16) prior autologous SCT while 3 patients did not have an autologous transplant due to a failed stem-cell collection, but failed other therapies. Thirty patients were in PR (n=26) or CR (n=4) at the time of SCT and 20 patients had stable or progressive disease. By day +100, 23 pts achieved CR (4 in CR pre-transplant, 15 of 26 in PR pretransplant; 4 of 20 in stable/PD); 17 PR, 7 died and 3 have already progressed. With median follow-up of 6.4 years (5-7.9 years), 16 patients are alive and 34 have died; 13 had NRM (cumulative incidence 26%) and 21 died of relapse. Three additional patients relapsed, but are currently alive with further therapies. The median survival is 2.3 years and the estimated 7-year overall and progression-free survival (PFS) rates were 34% (95 C.I. 21-47%) and 26% (95 C.I. 14-38%), respectively. The PFS curve showed an apparent plateau after 3 years, with no later relapses, suggesting potential cure. In multivariate analysis, adverse prognostic factors for survival included SCT not in remission, long duration of disease (> 5 years from diagnosis) and SCT from a female donor to a male recipient. Related and unrelated donor SCT had similar outcome. The 7 year PFS in 19 patients with none of these adverse factors was 47%. Chronic GVHD and achievement of CR after SCT were associated with improved outcome. *Conclusions.* Allogeneic SCT can result in long-term PFS in a subset of MM patients failing prior therapy and should be considered early after failure and preferably after achieving a response with salvage therapy. The treatment goal is to achieve CR as this is associated with better outcome. Relapsing disease is still the major cause of treatment failure. Additional strategies, such as maintenance therapy with novel agents or judicious use of donor lymphocyte infusions merit further investigation for converting PR to CR and reducing relapse risk.

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PEGFILGRASTIM REDUCES FEBRILE NEUTROPENIA, MUCOSITIS, DURATION OF HOSPITALIZATION AFTER AUTOLOGOUS PERIPHERAL BLOOD STEM CELL TRANSPLANTATION (PBSCT) FOR MALIGNANT LYMPHOPATHIES: REPORT OF 733 PBSCTS

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Granulocyte colony-stimulating factor (G-CSF) has been shown to decrease time to neutrophil recovery following PBSCT. Three products are available : 2 standard G-CSFs (filgrastim and lenograstim), and pegfilgrastim (PegG-CSF). In order to determine whether a single subcutaneous injection of Peg-G-CSF is as effective as a daily injection of standard G-CSF, in terms of haematological recovery, febrile neutropenic episodes (FN), antibiotic usage, hospitalization duration, mucositis, progressive free survival (PFS) and overall survival (OS), we retrospectively analyzed series of 604 patients having myeloma and lymphoma who underwent 733 PBSCT between 2000 and 2009 in our institution. Statistical analysis included univariate, Wilcoxon and χ^2 fisher tests, logrank test for PFS and OS, and Cox model for multivariate analysis. From January 2000 toDecember 2009, 377 patients received standard G-CSF (filgrastim or lenograstim), from May 2005 to December 2009, 330 patients received PegG-CSF. Since 2000, 26 patients did not receive G-CSF for any reasons. 455 PBSCTs have been performed for multiple myeloma (MM) after high dose melphalan, 211 for Non Hodgkin Lymphoma (NHL) and 67 for Hodgkin lymphoma (HL) with BEAM conditioning regimen. 121 patients underwent 2 or 3 PBSCT. The median of CD34 dose infused was 5.2×10^6/kg (1.2-30) with 96.2% of the grafts containing more than 2.5×10^{6}CD34/Kg. The median number of days of standard G-CSF given to reach an absolute neutrophil count (ANC) ≥500/mL was 9 days (4-29). Median time to neutrophil engraftment (ANC of 500/mL) was 11 days (5-30). The platelet recovery (platelet>20 000/mL) was 10 days (0-54) The platelet and RBC transfusion requirement are stastitically lower in the PegG-CSF group. As listed on the table I, we have analyzed the following parameters for all patients: number of FN and their beginning and duration, number of antibiotic lines, duration of hospitalization, duration of mucositis, and the percentage of grade III and IV mucositis.The same significantly differences are observed in MM, NHL and HL patients. The use of standard G-CSF or PegG-CSF does not modify OS at 1 and 5 years for both NHL and HL patients. In MM population PFS is unmodified. There is a trend of better OS in the PegG-CSF group compared to standard G-CSF : respectively OS at 1 year, 97% *vs.* 92%, OS at 5 years: 67% *vs.* 53% (pLog-rank=0.07) (pWilcoxon=0.05). Such a difference could be explained by the early use of bortezomib for induction therapy more frequently in PegG-CSF group. This feature has been analyzed. Among patients undergoing PBSCT, the use of Peg G-CSF seems to show an advantage in terms of duration of hospitalization and reduce the percentage of grade III, IV mucositis and the number of febrile neutropenic episodes.

Table.

Median, (range)	Peg G-CSF	Standard G-CSF	p	None G-CSF
N	330	377		26
FN rate (%)	74.5	80.9	<0.05	84.6
Duration of FN (days)	2 (0-27)	2 (0-18)	0.65	2 (0-13)
First day of FN (day)	5 (-3-16)	4 (-5-17)	<0.01	4 (0-11)
Number of antibiotic lines	2 (0-8)	2 (0-5)	<0.01	3 (0-4)
Duration of hospitalization (days)	18 (8-62)	19 (8-65)	<0.01	24 (18-35)
Duration of mucositis (days)	0 (0-75)	6 (0-60)	<0.01	7.5 (0-30)
Mucositis grade III, IV (%)	19.62	39.26	<0.01	ND

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QUANTIFICATION OF WT1 GENE EXPRESSION FOR MRD DETECTION AS PREDICTOR OF RELAPSE IN PATIENTS WITH AML AFTER ALLOGENEIC STEM CELL TRANSPLANTATION COMPARED TO FLOW CYTOMETRY AND CHIMERISM

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The detection of minimal residual disease (MRD) in AML patients after allogeneic stem cell transplantation (SCT) is important for the early detection of relapse and the selection of patients who can benefit from immunomodulation. Up to 65% of patients with AML lack a molecular marker useful for follow-up. The Wilms tumor gene (WT1) is overexpressed in more than 80% of AML patients at diagnosis, hence its utility as MRD marker. The objective of this study is to analyze the usefulness of WT1 quantification for MRD study and relapse prediction in patients with AML after allogeneic SCT and to compare the results with those from flow cytometry and chimerism. *Methods.* 174 samples were studied (69 BM and 105 PB) from 14 patients with AML (table 1), who underwent allogeneic SCT in a single center from 2003 and 2009. Quantification of WT1 expression was performed by quantitative RT-PCR (LightCycler 1.2) using cDNA obtained from 1 ug of RNA (Trizol®, Invitrogen). K562 cell line was used as calibrator and ABL as reference

gene (relative quantification). Cut-off values were defined as 0.5% in BM samples and 0.01% in PB. Results were correlated with those from flow cytometry and chimerism from the same samples. *Results.* WT1 overexpression correlated with the presence of disease before transplantation (Table 1). Post-transplantation, 6 of the 14 patients showed low and stable WT1 levels in BM and PB in the follow-up, remaining in CR (median follow-up 32 months, range 6-61). These 6 patients showed complete chimerism (CC) and negative MRD by flow cytometry. On the other hand, 8 from the 14 patients showed WT1 overexpression after SCT (Figure A): 6 of them showed, after initial low values, a significant increase in WT1 expression up to reaching positive levels in a median of 169 days (43-172), while 2 patients showed positive values continuously. Three patients showed positive levels only in PB samples, one only in BM and 4 both in BM and PB. From these 8 patients, 7 relapsed (1 with extramedullar disease who showed WT1 overexpression only in PB) in a median of 9 months after SCT (2.4-20). In one case, WT1 overexpression occured at the same time as relapse, while in the other 6 cases, relapse occured in a median of 137 days (47-438) after a significant increase in WT1 levels was seen in two consecutive samples. In these 7 relapsed patients, neither flow cytometry nor chimerism were earlier predictors of relapse. *Conclusions.* WT1 overexpression correlated with disease burden in AML patients after allogeneic SCT. In relapsed patients, relapse both medullar and extramedullar was anticipated sinificantly by WT1 overexpression compared to flow cytometry and chimerism. Quantification of WT1 overexpression by RT-PCR should be used for MRD detection in the follow-up after SCT in AML patients (not only in BM but also in PB for extramedullar disease detection) in order to facilitate immunossupresive therapy management and to select early DLI candidates.

Table 1. Caracteristics of 14 AML patients.

Case	Age	Sex	FAB	Karyotype	FLT3/NPM1	Pre-SCT status	TPH	Conditioning	Pre-SCT WT1
1	44	f	M1	+4,+8	na	Visible phase	HLA-id sibling	BuFlu	na
2	30	f	M5b	normal	na	CR		BuCy	na
3	41	m	M4	+8	na	CR		BuCy	negative
4	30	m	M1	normal	pos/neg	**CR, MRD+**		TBI-Cy	**positive**
5	39	m	RAEB-II	normal	neg/neg	**CR, MRD+**			**positive**
6	61	f	M5	complex	-	**Visible phase**			**positive**
7	19	m	M1	na	neg/neg	CR		BuFlu	na
8	41	m	M2	na	pos/pos	CR			negative
9	36	f	M0	na	pos/neg	CR			na
10	35	f	M4	normal	na	CR			negative
11	39	f	M1	normal	neg/na	**Aplasia**	MUD		**positive**
12	44	m	M2	complex	-	**CR, MRD+**		BuCy	**positive**
13	24	f	M4	complex	-	**Visible phase**	UCB/2ª donor (Dual)	BuFluCyATG	**positive**
14	48	f	M2	iso17	na	CR	Haplo	BuFlu (RIC)	positive

na: not available; PR: partial remision; CR: complete remision; MRD: minimal residual disease performed by flow cytometry

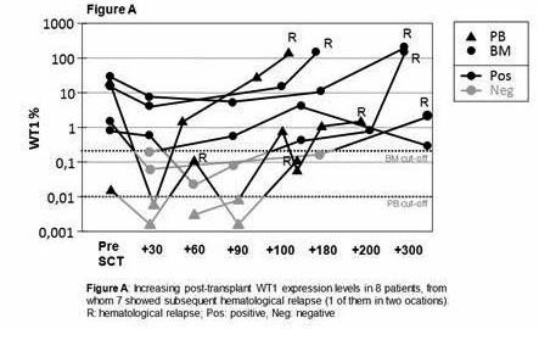

Figure A. Increasing post-transplant WT1 expression levels in 8 patients, from whom 7 showed subsequent hematological relapse (1 of them in two ocations). R: hematological relapse; Pos: positive, Neg: negative

Figure.

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DONOR-SPECIFIC DIFFERENCES IN LONG-TERM OUTCOMES OF TOTAL BODY IRRADIATION-BASED MYELOABLATIVE TRANSPLANTATION IN ADULTS WITH PHILADELPHIA-NEGATIVE ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. The graft-versus-leukemia effect in adult acute lymphoblastic leukemia (ALL) has now been definitely confirmed from the "sibling donor (SD) *vs.* no donor"comparisons. However, the role of stem cell transplantation (SCT) for Philadelphia chromosome (Ph)-negative acute lymphoblastic leukemia (ALL) remains unclear. In addition, "SD *vs.* no donor"approach is becoming outmoded, as in many studies those previously in a "no donor"category are now undergoing matched unrelated donor (URD)-SCT. *Aims.* We report long-term outcomes of total body irradiation-based myeloablative SCT in 292 consecutive adults with Ph-negative ALL who received transplants at our center between 1995 and 2008 (median follow-up of survivors, 84 months). This study focused on following questions: (1) How different are the outcomes of SCT according to the donor sources? (2) Which patients should be offered SCT using SD or URD? (3) Is there a role of autologous (AUTO)-SCT plus maintenance chemotherapy? *Methods.* Median age was 25 years (range, 15-63 years). Overall, 227 (77.7%) of 292 patients had one or more high-risk features, including adverse cytogenetics [t(4;11), t(8;14), complex (≥5 abnormalities), Ho-Tr], older age (≥35 years), high leukocyte counts (≥30×10^9/L for B-ALL, ≥100×10^9/L for T-ALL), or delayed first complete remission (CR1; >28 days). Two hundreds forty-one patients (82.5%) were transplanted in CR1; 22 (7.6%) in CR2; and 29 (9.9%) in advanced status. URD sources were classified as well-matched (WM), partially matched (PM), and mismatched (MM) based on recently published NMDP-CIBMTR guidelines. Of the whole group, donor grafts were SD (n=132), URD (n=68), and AUTO (n=92). All patients and donors provided written informed consent, and the treatment protocol was approved by the institutional review board of The Catholic University of Korea. *Results.* The cumulative incidence of relapse at 7 years was 50.4% for AUTO *vs.* 32.6% for SD, 19.4% for WM-URD, 32.3% for PM-URD, and 51.0% for MM-URD (HR=3.05 [1.82-5.10]; P<0.001). In multivariate analyses, other factors associated with relapse included transplant in beyond CR1 (P<0.001), T-lineage ALL (P=0.021), and adverse cytogenetics (P=0.033). The cumulative incidence of non-relapse mortality (NRM) at 7 years was 40.5% for MM-URD *vs.* 19.6% for SD, 20.3% for WM-URD, 15.8% for PM-URD, and 9.8% for AUTO (HR=3.10 [1.25-7.69]; P=0.015). Patients aged more than 35 years had a higher NRM (P=0.027). As a result, disease-free survival (DFS) at 7 years was inferior using AUTO (44.5%; HR=1.69 [1.14-2.51]; P=0.010) or MM-URD (26.3%; HR=2.03 [1.05-3.95]; P=0.036), while DFS from all other donor sources was approximately equivalent (53.5% for SD, 63.3% for WM-URD, and 57.0% for PM-URD). Transplant in beyond CR1 (P<0.001), older age (P=0.020), and adverse cytogenetics (P=0.041) were associated with poorer DFS. In a separate analysis including only patients in CR1, improved outcome was more pronounced in high-risk patients with a SD, WM-URD, or PM-URD. Interestingly, AUTO-SCT can induce durable DFS in standard-risk patients. *Summary/Conclusions.* Our long-term data suggest that outcomes are similar for transplantation using SD, WM-URD, or PM-URD sources, and these may be considered the best donor sources for patients with Ph-negative ALL, especially for those with high-risk features.

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THE SYNERGISTIC EFFECT OF HUMAN PARATHYROID HORMONE WITH MESENCHYMAL STEM CELLS ON ENHANCING HEMATOPOIESIS IN CORD BLOOD TRANSPLANTATION MODEL

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Background. Bone marrow niche and osteoblasts have important roles in maintenance and differentiation of hematopoietic stem cells(HSCs). Osteoblasts which are in contiguity with HSCs produce hematopoietic growth factors and insulin-like growth factors (IGF) and enhance hematopoiesis. Some investigators have reported parathyroid hormone (PTH) could activate osteoblast and hematopoiesis. *Aims.* We evaluated the effect of human PTH (hPTH) on engraftment or *in vivo* expansion of HSCs in a umbilical cord blood (UCB)-xenotransplantation model. In addition, the hPTH effect on hematopoietic activity of osteoblasts in co-transplantation of UCB derived mesenchymal stem cells (MSCs) and the role of hPTH in enhancing the expression of cell adhesion molecules was evaluated. *Methods.* Female NOD/SCID mice received sublethal total body irradiation with a single dose of 250 cGy. After 18 to 24 hours of irradiation, 1×10^7 human UCB-derived mononuclear cells (MNCs) and 5×10^6 human UCB-derived MSCs were infused via the tail vein. The mice were randomly divided into the three groups: Group 1 (n=3) received MNCs only, Group 2 (n=3) received MNCs only and then treated with hPTH, Group 3 received MNCs and MSCs, and then treated with hPTH. The mice of Group 2 and Group 3 were injected intraperitoneally with 40 μ/kg/day of recombinant hPTH 5 times/week during the first 4 weeks after transplantation. At the fourth, sixth and

seventh week after transplantation, blood samples were drawn for complete blood count (CBC) and then both femora and tibiae were excised and marrows were aspirated. Assessment of the bone marrow cellularity, flow cytometric analysis for human hematopoietic progenitor cell markers and cell adhesion molecules as CXCR4, VLA-4, VLA-5 were performed at the seventh week. *Results.* Engraftment was performed in all mice of three groups. At the fourth, sixth and seventh week after transplantation, there were no statistical differences of white blood cell (WBC), red blood cell (RBC) and platelet counts among 3 groups. Bone marrow cellularity was approximately 20% in Group 1, but 70-80% in Group 2 and Group 3. Although hPTH-treated groups showed higher cellularity, MSC co-transplantation had no effect on bone marrow cellularity. The flow cytometric analysis showed that the proportion of myeloid(human CD13, CD33) and lymphoid(human CD19) lineages were predominantly higher in Group 3. In particular, the percentage of total human hematopoietic progenitor cells in Group 2 and 3 was larger than in Group 1. The proportion of CXCR4 in Group 3 was larger than in Group 1 and 2. However, the differences did not reach statistical significance among the 3 groups. *Summary/Conclusion.* This study showed a possible synergistic effect of MSCs and hPTH for enhancing the proportion of human hematopoietic progenitor cells in UCB xenotransplantation model. These findings might suggest that the differentiated osteoblasts from co-transplanted MSCs, resulted in hematopoietic progenitor cell expansion in UCB-xenotransplantation model.

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CLINICAL FEATURES AND OUTCOME OF 2009-H1N1 INFLUENZA AMONG ADULT ALLOGENEIC HEMATOPOETIC STEM CELL TRANSPLANT RECIPIENTS

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Background. Recently, a new type of influenza virus, the 2009 H1N1-Influenza A virus (H1N1-influenza) has been involved in a worldwide pandemic outbreak. *Aims.* The impact of H1N1-influenza in allo-HSCT recipients is unknown. The aim of this prospective study was to define the features and outcome of this novel infection among adult allo-HSCT recipients. *Methods.* From May 2009 through February 2010, all adult allo-HSCT patients who presented with respiratory viral symptoms (RVS) were screened for the presence of H1N1-influenza virus. Specimens were taken from nasopharyngeal swabs or bronchoalveolar lavage. Laboratory confirmation was based on a H1N1-influenza specific real-time RT-PCR. Oseltamivir resistance was assessed in patients who had a clinical severe course. Complete clinical and biological chart reviews were performed for all cases. *Results.* In all, 9 patients out of 248 had a confirmed H1N1-influenza diagnosis, giving an overall incidence of nearly 4% over the study period. At time of infection, all patients but one were in CR and had full donor chimerism. Infected patients were heavily pretreated before allo-HSCT. Close contact with children and young adults was the most frequently suspected transmission mode (4 out of 9). With regards to their immunological status, all patients but one were vaccinated against seasonal influenza and 5 against H1N1-influenza. All infected patients presented upper respiratory tract symptoms (URT) with fever and cough. Lower respiratory tract symptoms (LRT) were present in 4 cases. The duration of symptoms before diagnosis confirmation ranged between 0 to 15 days. Other reported manifestations were runny nose, sore throat, muscular pain and dyspnea. Chest radiographies were available in 4 patients at presentation and abnormalities were detected in 2 cases. All patients received oseltamivir (initiated 0 to 8 days after symptoms) for a duration of 5 days. 4 patients were hospitalized. 5 patients had other associated viral or bacterial pathogens in respiratory secretions, and 7 received systemic antibiotics in addition to antiviral treatment. 3 patients with significant comorbidities and active chronic extensive GVHD had prolonged hospitalization and required mechanical ventilation. In addition to oseltamivir, IV zanamivir was given for a period ranging from 8 to 15 days. Interestingly, these patients had a prolonged viral shedding as assessed by serial virologic testing. Sequencing of the neuraminidase gene showed an oseltamivir resistant strain in 2 patients. Among them, one patient died within 60 days after diagnosis due to sepsis and respiratory failure for an overall H1N1-influenza attributable death around 11%. *Conclusions.* This large comprehensive single centre analysis suggest that although most allo-HSCT recipients had mild symptoms from H1N1-influenza, high immunosuppression and emergence of oseltamivir resistant strains are still a matter of concern. Although H1N1-influenza vaccination might have prevented infections or decreased the severity of disease in many patients, our study suggests that the efficacy of this vaccine in eliciting a protective antibody response is still poor in allo-HSCT patients. Close contact with children and young adults was the most frequently reported risk factor for contamination supporting the need for considering vaccination and monitoring of family members and close contacts.

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DEVELOPING A PREDICTING SCORE FOR SUBOPTIMAL MOBILIZATION IN LYMPHOMA PATIENTS

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Background. Autologous stem cell transplantation (ASCT) is a potentially curative treatment for lymphoma. A suboptimal mobilization, defined as a collection of 2 to 5×10^6 $CD34^+$ cells/kg, could impair the outcome of high dose treatment, even more if a tandem transplant is planned. New agents were recently developed which may improve the rate of SC mobilization. *Aims.* To identify a predictive score for suboptimal mobilization defined on a large series of lymphoma pts candidates to ASCT according to factors influencing SC mobilization outcome as previously reported (Morello, ASH 2009). *Patients and methods.* A total of 415 attempts of PBSC collection, consecutively performed at 7 Italian centres in 388 pts affected by lymphoma, were previously analysed. A collection of 2 to 5×10^6 $CD34^+$ cells/kg was defined as "suboptimal mobilization". The following parameters were analysed for correlation with poor mobilization: lymphoma diagnosis, disease status at mobilization, type of mobilizing chemotherapy, bone marrow infiltration at collection, n° of previous lines of therapy, prior use of fludarabine, alkylating agents or radiotherapy. The ratio between circulating $CD34^+$cells/μL and total WBC/μL on the first day of $CD34^+$ count (SCratio) was also analysed, trying to predict mobilization failure. Multivariate statistical analyses by logistic regression was performed with NCSS 2007. *Results.* The identified variables at the multivariate analysis affecting suboptimal mobilization were: age >60 (P=0.03, OR 2.26), previous treatment with alkylating agents (P=0.009, OR 3.41), 1.5≤CD34/μl<125 (P=0.0001, OR 5.24), 0.0006≤SCratio<0.004 (P=0.005 OR 2.75), mobilization with cytarabine (P=0.015 OR 0.32). Based on the regression coefficients of the multivariate analysis for each variable, a scoring system for suboptimal mobilization was established as following: Age >60=1, Alkylating agents=1, 1.5≤CD34/μL<125=2, 0.0006≤SCratio<0.004=1, Cytarabine=-2. A score for each specific risk profile was calculated by summing integer points. The score had a theoretical range between -2 and 5. A score of 3, 4 and 5 has a probability of suboptimal mobilization of 50, 69 and 83% respectively. *Conclusions.* This prognostic score should be confirmed by a validation set, currently under investigation, and thereafter a prospective trial will be implemented in order to rationally explore the use of new mobilizing agents in patients with lymphoma undergoing SC mobilization with chemotherapy and G-CSF.

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FACTORS DETERMINING T CELL DONOR CHIMERISM AFTER AN ALEMTUZUMAB BASED REDUCED INTENSITY ALLOGRAFT

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Background. Reduced intensity conditioned (RIC) allogeneic transplants are widely used to deliver a graft-versus-leukaemia (GvL) effect in older patients with haematological malignancies. It is postulated that the presence of mixed chimerism in the T lymphocyte compartment post-transplant is indicative of bidirectional tolerance and therefore a predisposing factor for disease relapse. However the factors determining donor T cell chimerism after an alemtuzumab based RIC allograft have not been systematically studied to date. *Aims.* We therefore analysed pre-transplant characteristics in 140 patients undergoing a RIC allograft for a haematological malignancy in order to identify factors determining post transplant chimerism. *Methods.* 71 patients were transplanted for a

myeloid malignancy (acute myeloid leukaemia (n=44), chronic myeloid leukaemia (n=10) or myelodysplasia (n=17)) and 69 for a lymphoid malignancy (Non Hodgkin's (n=57) or Hodgkin's lymphoma (n=12)) The conditioning regimen used was Fludarabine and melphalan or busulphan for patients with a myeloid malignancy and BEAM (Carmustine, Etoposide, Cytarabine, Melphalan) for patients with a lymphoid malignancy. 91 patients received a transplant from a sibling donor and 49 from an unrelated donor. The median follow up was 23 months (3-50 months). Pretransplant parameters considered were age, sex, stem cell dose, transplant type(sibling *vs.* MUD), stem cell source (BM *vs.* PBSC), CD34 dose, CD3 count , disease type(myeloid *vs.* lymphoid), and disease status at transplant (CR *vs.* not CR). RESULTS In univariate analysis transplantation from an unrelated donor, a higher CD3 count in the stem cell inoculum and advanced patient age were associated with a higher rate of full donor T cell chimerism (FDTC) on day+90. Multivariate logistic regression of the above baseline covariates found that stem cell source (sibling v unrelated) and patient age predicted FDTC at day 90. FDTC at 90 days did not impact on overall survival, disease free survival, or chronic GvHD. In contrast FDTC at day 90 was associated with an increased risk of acute GvHD. *Conclusions.* This study has identified factors influencing chimerism status after an alemtuzumab based RIC allograft. Whilst acquisition of FDTC was associated with an increased risk of acute GVHD no effect on overall survival or disease free survival was noted. Confirmatory studies in a larger cohort of patients will be important but our data are consistent with the hypothesis that the GvL effect may be mediated through a T cell independent mechanism in the setting of an alemtuzumab based RIC allograft.

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LOW DOSE METHOTREXATE COMBINED WITH LOW DOSE METHYLPREDNISOLONE AS FIRST-LINE THERAPY FOR THE TREATMENT OF ACUTE GRAFT-VERSUS-HOST DISEASE - SAFETY AND FEASIBILITY STUDY

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Background. The current standard therapy for aGVHD is glucocorticoid methylprednisolone 2mg/kg/d, but it still has drawbacks. Recent studies have suggested that low dose of MTX, in addition to having anti-mitotic effects, can induce a sustained suppression of T-cell activation, supporting its use in GVHD therapy. Our recently published data found that low dose of methotrexate can be used to treat different types of GVHD including aGVHD, cGVHD and post-DLI GVHD as salvage therapy. *Aims.* The current study was initiated to evaluate the safety and both the efficacy of low dose of methotrexate combined with low dose of methylprednisolone as first line therapy in the treatment of acute graft-versus-host disease (aGVHD) after allogeneic hematopoietic stem cell transplantation (allo-HSCT). *Methods.* The study was approved by the Institutional Review Board of the Peking University Institute of Hematology. All included patients were informed and signed an informed consent form. Patients who had not received drug treatments for aGVHD and whose peripheral blood white blood cell counts were higher than 1.5×10^9/L were selected. From May 2007 to June 2008, 32 patients received intravenous MTX at a dose of 10 mg or oral MTX at a dose of 15 mg every 3-7 days (repeated at day three after the first dose and then at a weekly interval) combined with low dose of methylprednisolone (started with 0.5mg/kg/d, and dose was reduced by half after 5-7 days) until a complete or partial response was achieved, or until treatment failure or intolerable side effects were found. Patients were observed for 3-5 days and would be switched to second line treatment (Daclizumab) if no response to first line therapy. *Results.* The median time from HSCT to the start of MTX was 32 days. The median number of MTX administrations was four (range, 2-6). Median time to achieve maximal response (CR or PR) was 5 days. By day 30 after drug administration, accumulated MP dose was 5.78 mg/kg. The overall response rate was 81% (26/32 patients). The response rate for GVHD involving various organs was 88% (23/26) in skin, 75% (3/4) in liver, 81% (9/11) in gut. Six of 32 (18%) patients required Daclizumab as second-line treatment. Grade 3 toxicities occurred in only 3 patients presenting cytopenias. Eighteen of 32 (56%) patients developed cGVHD. Two-year cumulative incidence of leukemia relapse was 7%. Two-year cumulative incidence of TRM was 11%. Twenty-seven patients (84%) remain alive without leukemia relapse with a median survival of 682 days from onset of aGVHD and 699 days from HSCT. The estimated survival at 2 years was 77%. *Summary/Conclusions.* Our study showed that low-dose MTX combined with low dose of methylprednisolone is effective and safe. There are maybe synergistic effect between methotrexate and methylprednisolone. The combination regimen might be eligible for alternative forms of first-line treatment of aGVHD and it is especially preferential for those high risk patients of relapse. However, a randomized, controlled study is needed to compare the results of this new regimen and the standard therapy with methylprednisolone (2 mg/kg/d).

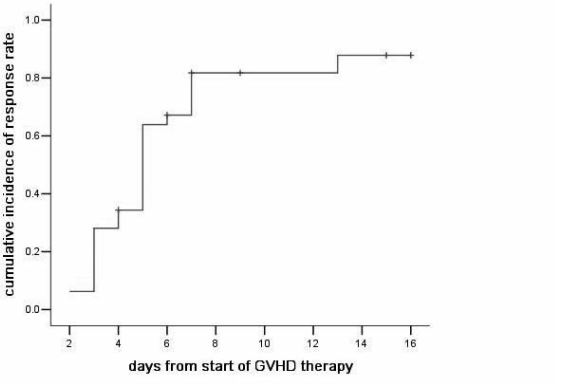

Figure. Cumulative incidence of acute GVHD response.

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KINETICS OF IMMUNE CELL RECONSTITUTION FOLLOWING SUCCESSFUL MIXED CHIMERISM BY NONMYELOABLATIVE BONE MARROW TRANSPLANTATION IN MICE

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Nonmyeloablative bone marrow transplantation (BMT) utilizes low-doses of radiation and relatively nontoxic conditioning. Allogeneic mixed chimeras following nonmyeloablative conditioning showed donor-specific tolerance and superior immunocompetence. In this study, we induced stable allogeneic mixed chimerism by host natural killer (NK) cell depletion and T cell-depleted BM grafts in a major histocompatibility complex (MHC)-mismatched murine model and analyzed the kinetics of donor and recipient engraftment in the weeks following transplantation. Donor BM cells were well engrafted and stabilized without graft-versus-host disease (GVHD) as early as one week post-BMT. Donor-derived thymic T cells were reconstituted four weeks after BMT; however, the emergence of newly developed T cells was obvious at the periphery as early as two weeks after BMT. Also, NKT cells developed four weeks after BMT, and the ratio of recipient- to donor-derived NKT cells was the same twelve weeks after BMT. Interestingly, the emergence of donor-derived antigen presenting cells (APCs) such as dendritic cells (DCs) and B cells was noted as early as one week after BMT, but changed only modestly between weeks 2 and 12 after BMT. Here, we report a kinetic model of the development of donor- and recipient-originated hematopoietic cells in a mouse model of allogeneic mixed chimerism during the early post-transplant period. These data expand our understanding of immune cell reconstitution at early time points after nonmyeloablative BMT and may be used to develop guidelines for the treatment of patients post-BMT.

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18F-FLUORODEOXYGLUCOSE POSITRON EMISSION TOMOGRAPHY IN THE DIAGNOSIS OF INTESTINAL GRAFT VERSUS HOST DISEASE: A NEW TOOL?

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Background. The diagnosis of gastrointestinal graft-versus-host disease (GVHD) after allogeneic stem cell transplantation is usually assessed by

clinical symptoms and histological analyses after digestive endoscopy. This approach is frequently not satisfactory because of its lack of specificity. A murin transplantation model has suggested that inflammatory activity associated with intestinal GVHD can be assessed by 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) *Aim.* We evaluated the relevance of FDG-PET in the diagnosis of intestinal GVHD in transplanted patients. *Methods.* Between February 2008 and March 2009, patients with a suspected diagnosis of gastrointestinal GVHD after haematopoietic stem cell transplantation (HSCT) were enrolled in our study. They were examined by FDG-PET and digestive endoscopy (gastroduodenal fibroscopy, rectosigmoidoscopy and colonoscopy when feasible) with biopsy. Stool samples were screened for bacterial, viral, mycological and parasitic gastrointestinal infections. Patients were systematically screened for adenovirus and cytomegalovirus (CMV) infections by specific PCR in the blood and immunofluorescence in digestive biopsies. *Results.* Eleven patients with clinical symptoms of GVHD in a median time of 35 days (4-392) after HSCT were tested. Two patients had a stage 2 digestive GVHD and 9 patients a stage 3-4. None of them was positive for viral, mycological or parasitic infections in the stool, 6 were colonized with an intestinal bacteria but all were negative for clostridium difficile toxin. One patient presented a CMV colitis based on the biopsy and blood samples. FDG-PET was performed in a median time of 6 days after the beginning of the symptoms (1-159). Seven patients presented a FDG uptake of the gut. All of the 11 patients had sigmoid biopsies, 6 had staged biopsies of all colon and 4 gastroduodenal biopsies. For 6 patients, histological and FDG-PET results were concordant (5 were both positive and 1 both negative). In 3 patients, the FDG-PET showed no uptake whereas the histological analysis was positive. For the 2 remaining patients, FDG uptake was localized in the ileum and therefore not accessible to biopsy. *Conclusion.* FDG-PET is a non invasive tool that can be useful in some patients with suspected intestinal GVHD. This approach may be interesting to guide biopsy when the diagnosis is difficult with the usual assessment, in particularly in the small intestinal localization.

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STRATEGIC DESIGN OF THE HELLENIC UMBILICAL CORD BLOOD BANK NETWORK

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Background. Umbilical Cord Blood (UCB) has emerged as a widely accepted source of Hematopoietic Stem Cells (HSCs) for allogeneic transplantations thus leading to a number increase of public UCB banks worldwide. In Greece the development of such a UCB bank network is in its infancy, while there are still several issues to be regulated. *Aims.* This research work presents a methodological framework for the systemic design of the Greek National UCB bank network that may be applicable to other countries with no national UCB bank and relatively isolated population. We aimed at the determination of the UCB banks capacity, the key design parameter, including the supplementary cost estimations, banks' location, and personnel planning. *Methods.* The main dataset (histocombatibility data) encompasses: (i) the haplotypes (HLA-A, -B, -DR, all in low resolution) and their frequencies of 7,710 Greek bone marrow and UCB donors (BMDW registry), and (ii) the genotypes (HLA-A, -B in low resolution, and HLA-DR in high resolution) of 410 Greek donors (Greek National Histocombatibility Center). Following the statistical analysis of the available data, extensive Monte Carlo simulation experiments were conducted to determine the quantitative relationship between the inventory level of a national UCB network (capacity planning) and the probability that a transplant candidate finds a matching unit (service level). *Results.* The pivotal result is a methodology for determining the relationship between the inventory level and the service level of a national UCB bank. Specifically, the simulation process outcomes reveal that an inventory of 10,000 cryopreserved UCB units provides a 96% likelihood that a Greek transplant candidate finds a matching unit (4 out of 6 HLA-match/ HLA-A, -B, -DRB1). Taking into account a 60% discard proportion for the donations - according to literature and applied practices - and a realistic annual collection rate of 3,125 units, the desired inventory level could be reached in approximately 8 years. The bank network design documented the need for operating two public UCB banks (dipolar system) located in Athens and Thessaloniki, respectively. The necessary personnel (administrative officers/medical doctors, technicians, employees, and a trainer) are 11 persons per bank during the inventory build-up period and 7 persons per bank during the regular operating period. The initial investment cost is approximately €1.1M, while the inventory build-up cost is estimated at € 12.4M. Additionally, the annual average operational cost (steady-state) is about €1.2M. This cost could be easily balanced by a fee of approximately €7,000 received for every unit used for transplantation, paid by the relative social health insurance companies. *Summary/Conclusions.* The findings document the importance of developing a national UCB bank network, the subsequent improvement in provided health, and economic ramifications, while providing the necessary input for the NHS to decide on developing or not a UCB bank. Furthermore, the entire network's design is feasible and economic sustainable, according to the NHS economic capabilities, the national and European legislation, and the applied practices worldwide. Finally, the support from the Greek government and sponsors are of critical importance for its success.

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ELEVATED SERUM FERRITIN AND MAJOR TRANSPLANT-RELATED COMPLICATIONS AFTER HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. Iron overload (IO), mainly from red cell transfusions, is a frequent condition in hematopoietic stem cell transplantation (HSCT). IO is associated with free radical generation and tissue damage which can increase toxic and infectious events after HSCT. We retrospectively evaluated the clinical impact of pretransplantation iron status on early transplant related toxicity. Patients and *Methods.* The charts of 224 (110 M, 114 F, median age 43 years, range 17-70 years) patients who underwent HSCT [142 autologous (auto) and 82 allogeneic (allo)] were reviewed. Serum ferritin, a surrogate marker of iron overload, was measured before the beginning of the conditioning regimen. The range of diagnoses included 83 patients with acute myeloid leukemia, 35 with acute lymphoblastic leukemia, 2 with myelodysplasia, 45 with myeloma, 59 with lymphoma. In the allo group, myeloablative conditioning was employed for 63 pts and reduced intensity conditioning for 20 pts. The median pretransplant serum ferritin of the 224 patients was 720 ng/mL with values ranging from 20 to 9255 ng/mL. We set 800 ng/mL as the cut-off value for pre-transplant serum ferritin, obtaining a group of 143 patients with ferritin levels <800 ng/mL (low ferritin group) and a group of 81 above the cut-off level (high ferritin group). The effect of elevated pretransplant ferritin on the incidence of mucositis, blood stream infections, day 100 mortality, acute GvHD and invasive fungal disease was assessed. Student's t-test or the Mann-Whitney test was performed for comparisons of means. *Results.* The high ferritin group showed a higher incidence of grade 3 and 4 mucositis than the low ferritin group after both auto (30 % *vs.* 15%, P=0.05) and allo transplantation (34% *vs.* 15%, P=0.03). At univariate analysis, the incidence of bloodstream infections was significantly lower in the low ferritin group after auto (15% *vs.* 30%, P=0.05) and allo HSCT (25% *vs.* 40%, P=0.05). In the autologous group, the high ferritin group had increased day 100 mortality (5% *vs.* 1%, P=0.06). In allogeneic group, the high ferritin group had increased day 100 mortality (due to hepatic veno-occlusive disease, pneumonitis and severe endothelial leakage syndrome) (20 *vs.* 8%, P=0.03), increased acute GvHD grade II-IV (57 % *vs.* 25%, P=0.02), increased invasive fungal disease (18% *vs.* 5 %, P=0.05). The time to engraftment was shorter in the low ferritin (median: 13 days, range: 11-16 days) than the high ferritin group (15 days, 13-23 days) after allogeneic HSCT (P=0.05). *Conclusions.* Pre-transplant serum ferritin was a predictor for major transplant-related complications, especially after allogeneic HSCT. Our results need to be validated by further prospective studies. Methods for modifying IO in candidate patients for HSCT should be developed.

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EBMT RISK SCORE IN ADDITION TO HLA-DRB11 AND HLA-A3 PREDICTS NON-RELAPSE MORTALITY IN A SINGLE CENTER ANALYSIS

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Background. Matched related donor peripheral blood stem cell transplantation (MRD-PBSCT) is associated with a low incidence of non relapse mortality (NRM) and graft-versus-host-disease (GvHD). EBMT risk score for outcome after PBSCT has been shown to be predictive across all disease categories for overall survival (OS) and NRM (Gratwohl *et al.*, Cancer 2009). *Aims.* We have analysed the influence of the EBMT risk score and other patient specific factors in patients undergoing MRD-PBSCT in a single center. The endpoints of this study were OS, relapse incidence (RI), NRM and incidence of acute and chronic GvHD. *Methods.* The outcome of 97 patients undergoing MRD- PBSCT after myeloablative conditioning without T-cell depletion and a post-grafting immunosuppression with cyclosporine and short course MTX was analyzed. The EBMT risk score (disease stage, time from diagnosis to transplant, patient`s age and donor/recipient sex combination) was evaluated and type of disease, conditioning regimen, donor's age, CMV status, and HLA-type added into a multivariate analysis. *Results.* After a median follow up of 42 months OS was 66%, NRM 18% and RI 22% The incidence of acute GvHD grade III-IV was 18%, chronic GvHD was 55% (37% limited and 18% extensive). Main causes of death were: relapse, GvHD and infectious complications in 15, 12, and 6 patients, respectively. In multivariate analysis, patients with a risk score ≥ 4 had a higher NRM (P=0.004), while patients with HLA-DRB1-11 and HLA-A3 had a higher NRM and a poorer OS (P<0.002). Patients with a higher risk score had more skin GvHD grade II-IV (P=0.002), while patients with HLA-C7 had more overall GvHD (P=0.0014). No significant risk factors for the development of chronic GvHD and RI were detected in this analysis. *Conclusion.* In conclusion, EBMT risk score was a predictive factor for NRM and GvHD in a group of patients undergoing MRD-PBSCT in a single center evaluation. In addition, the presence of HLA-C7 was associated with more overall GvHD, while HLA-DRB1-11 and HLA-A3 had a poorer outcome.

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ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT) IN CHRONIC MYELOID LEUKAEMIA (CML): LONG TERM RESULTS

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Background. Allogeneic HSCT is recognized as being the only effective treatment in the CML with cure. We report our experiment of this procedure with a comparative study between myéloablative and reduced intensity conditioning regimen. *Material and methods.* From July 98 to June 2009, 274 patients (pts) with CML underwent allogeneic HSCT with HLA identical sibling donors. The myeloablative stem cell transplantation (MST) based on Tushka protocol was employed in 101 pts including 56 in first chronic phase(CP), 39 in accelerated phase (AP) and 6 in blast crisis(BC). The prevention of the graft *vs.* host (GVHD) associated ciclosporine and méthotréxate according to Seattle protocol. The non myeloablative SCT (NST) based on the use of Fludarabine and Busulfan was employed in 173 pts including 149 pts in first CP and 24 in AP. The GVHD prophylaxis consisted of cyclosporine with mycophenolate mofetil. The median age of the pts at transplant for MST and NST is 24 years (4-44) and 36 years (18-55) respectively with a significant difference (P<0.01). The sex-ratio is 1,2 and 0,96 respectively; interval diagnosis was longer in MST pts: 15 months (3-82) than in NST pts: 11 months (4-58) (P<0.01). The grafts used are peripheral blood stem cells (MST=90, NST=173), bone marrow (9 MST) and umbilical cord blood (2 MST). *Results.* Aplasia is occurred in 76 pts (44%) NST group and all pts of MST group (100%), (P<10-8). The incidence of acute GVHD was the same in the MST and NST group (P=0,35) with respectively 48 pts (47,5%) and 72 pts (42,8%). The incidence of chronic GVHD was also the same (P=0.14), 58 MST pts (63.7%) and 114 NST pts (71.2%). The cytomegalovirus (CMV) infection was seen in 23 MST pts (22.7%) and 28 NST pts (16%) without significant difference (P=0.16). Relapse of disease occurred in 15 MST pts (14.8%) and complete remission was obtained in 5 pts (immunosuppression discontinuation :1, DLI: 2 and Imatinib: 2); In the group NST 31 pts relapsed (17,9%) and in 15 pts remission obtained after salvage treatment (immunosupression discontinuation: 8, DLI: 3 and 2d myeloablative allograft: 4) (P=0.7). At December 2009, 56 MST pts (55.4%) and 109 NST pts (63%) are alive with a median follow-up in 69 months and 64 months respectively. The Overall Survival (OS) is 41.9% for the MST group and 56,8% for NST group (P<0.001); Event Free Survival (EFS) is 36.7% and 49.5% respectively with significant difference (P<0.001). *Conclusion.* No differences were observed between MST and NST group concerning acute and chronic GHVD, CMV infection and incidence of relapse. However in long term results, OS and EFS are better in NST group than MST group.

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SIGNIFICANCE OF INCREASED BONE MARROW BLASTS AFTER MYELOID ENGRAFTMENT IN ADULT PATIENTS UNDERGOING UNRELATED CORD BLOOD TRANSPLANTATION

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Background. Abnormal expansion of naïve B-lymphocytes has been reported after unrelated cord blood transplantation (UCBT). In some cases, blastic-appearing lymphocytes may be difficult to distinguish from malignant blasts. *Aims.* Analyze the incidence and significance of an increase in the bone marrow (BM) blast count by cytomorphology after UCBT. *Methods.* Between May 2001 and February 2010, BM samples of patients undergoing UCBT in a single institution were evaluated by cytomorphology and multi-parametric flow cytometry (MPFC). All patients included in the study had myeloid engraftment. BM samples were stained using May-Grünwald-Giemsa stain and viewed under light microscope. Immature B-lymphocytes and leukemic blast cells were discriminated by MPFC on the basis of the normal B-cell maturation using the combination of CD10, CD20, CD19, CD34 and CD45 antigen surface markers. Malignant blastic cells were characterized according to the blast phenotype at diagnosis. BM samples were taken from day +40 to day +365 after UCBT. Chimerism analyses of BM samples using multiplex PCR amplification of short tandem repeat-markers (STR) were also performed. The diagnostic sensitivity and specificity of cytomorphology for the detection of BM leukemia was determined using the MPFC as validation test. *Results.* One-hundred and nineteen samples from 75 patients were analyzed by cytomorphology and MPFC. Median age at UCBT was 32 years (range, 15 to 64), 42 (56%) patients were male and underlying diseases were acute lymphoblastic leukemia (ALL) in 32 (43%), acute myeloid leukemia (AML) in 25 (33%), and other malignancies in the remaining 18 (24%). Non-myeloablative conditioning regimens were used in 9 (12%) patients. The median percentage of blasts in BM samples by cytomorphology was 4% (range, 0% to 95%), and 53 (45%) showed an increase of blasts (≥5%). Thirty-one (58%) of the 53 samples with ≥ 5% blasts by cytomorphology were characterized as non-malignant cells by MPFC. In those samples, the median percentage of blasts by cytomorphology and MPFC were 7% (range, 5% to 14%) and 4% (range, 0.5% to 10%), respectively. Among those 31 samples, the median percentage of lymphocytes by MPFC was 26% (range, 8% to 56%), predominantly corresponding to B-lymphocytes in 28 (90%) cases. In all these cases, STR analyses showed full-donor chimerism. Twenty-two (42%) of the 53 samples with ≥5% blasts by cytomorphology were characterized as leukemic cells by MPFC, with a median percentage of blasts by cytomorphology and MPFC of 50% (range, 9-95%) and 35% (range, 3-93%), respectively. Relapses corresponded to ALL in 13 cases and to AML in 9 cases. No cases of leukemic relapse were detected by MPFC when BM blast count by cytomorphology was <5%. Diagnostic sensitivity, specificity, and positive predictive value of cytomorphology for the detection of BM leukemia were 100%, 75%, and 58%, respectively. ì*Conclusions.* The observation of ≥ 5% blasts by cytomorphology in BM samples after myeloid engraftment in adult patients undergoing UCBT is relatively frequent. However, this finding will indicate in most cases an increase of immature B-lymphocytes that should be distinguished from malignant blasts using MPFC.

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CYTOKINE PRODUCTION BY GRAFT CELLS IN RESPONSE TO PATIENT'S ANTIGENS MAY PREDICT THE OCCURRENCE OF ACUTE GRAFT VERSUS HOST DISEASE IN ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION FROM A SIBLING

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Background. Allogeneic hematopoietic stem cell (HSC) transplantation whether bone marrow (BM) or peripheral blood (PBSCT) is a widely used therapeutic modality. Graft *vs.* host disease (GVHD) continues to be the main concern of transplanters. There is substantial evidence to implicate that cytokines play a major role in GVHD induction and grade. GVHD was reported to be associated with increased production of IFNgamma and IL10. IL 10 cytokine level and gene polymorphism have been used as predictors of acute GVHD (aGVHD). IFNgamma was also reported to have a protective role if administered at the time of transplantation possibly via induction of T regulatory cells and immunological tolerance. *Aims.* The aim of this work was to develop an experimental setup to mimic the response of the immune cells of the graft to the host antigens expressed by cytokine production. The final aim was to correlate the cytokine pattern to the development of aGVHD and verify if this pattern could possibly predict the occurrence of aGVHD. *Methods.* The study included 46 patients who received allogeneic PBSCT from an identical sibling. Under informed consent, a sample was obtained from the patient before conditioning, mononuclear cells separated and cryopreserved. On the day of transplant, the cryopreserved cells were thawed, mitomycin treated to serve as stimulators while the mononuclear cells of the graft served as the responders in a mixed lymphocyte culture setup. After 3 days culture, supernatant was collected and stored at -80 degree C till tested. IFNgamma and IL10 were measured by microbead array technology using luminex 200. Patients were followed up and development of aGVHD was recorded. *Results.* Of the 46 patients, 14 developed aGVHD. In the culture supernatant, cytokines were below the detection limit in 26/32 of cases that did not and in 3/14 of those who developed aGVHD. The level of cytokines in the other cases varied widely. Of the 11 cases that showed cytokine production, 4 produced IFNgamma only and one produced IL10 only. The other 6 cases produced both; IL10 was higher in one case; in the other 5 cases, IFNgamma was much higher. In the 6/32 cases without GVHD, IL10 only was detected in 3 cases; the other 3 cases showed much higher level of IL10 than IFNgamma. At a cutoff level of 15.9 pg/mL, IFNgamma was predictive of aGVHD with a sensitivity of 64.3%, specificity of 96.8% and a total accuracy of 80.4%. At a cutoff of 2.27pg/mL, IL10 showed a sensitivity of 50%, specificity of 80.6% and a total accuracy of 71.1%. At a cutoff of 1.13, IFNγ/IL10 ratio showed a sensitivity of 85.7%, specificity of 83.3% and a total accuracy of 84.6%. *Conclusions. In vitro* cytokine production by graft immune cells in response to host antigens is extremely variable; it may serve as a surrogate system of the immune reaction following allogeneic stem cell transplantation. IFNgamma production apparently reflects potential development of aGVHD while IL10 production is apparently protective. When both are produced the IFNgamma/IL10 ratio is more informative than either alone.

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ISOLATED EXTRAMEDULLARY RELAPSES AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION FOR ACUTE AND CHRONIC LEUKEMIAS: A SINGLE INSTITUTE EXPERIENCE WITH 545 PATIENTS

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Background. Isolated extramedullary relapses (IER) of leukemias are rare after allogeneic hematopoietic stem cell transplantation (alloHSCT) and the data regarding their incidence in larger series of patients (pts) and long-term outcome are scarce. *Aims and methods.* We retrospectively analysed incidence, clinical presentations, treatment options and long-term outcome of this mode of leukemia recurrence in a cohort of 545 consecutive pts (123 with ALL, 201 with AML, 208 with CML, 13 with CLL) who underwent alloHSCT in our institution between June 1993 and December 2007. 81 pts (34 with ALL, 28 with AML, 17 with CML, 2 with CLL) relapsed (any site). *Results.* 9 (11%) out of all pts who relapsed (4 with B-line ALL, 4 with AML, 1 with CML, F/M 4/5, median age 29 years, range 28 - 44 years) developed IER after a median time of 17 months (mts) (range, 8 - 80 mts) following alloHSCT. We revealed complete donor chimerism in 7/9 studied pts. 4 pts (3 with ALL, 1 with AML) developed skin and/or subcutaneous tissue infiltrates; in one of them (patient with ALL) leukemic tumor of the peritibial soft tissues was additionally observed. Other sites of IER included (No. of cases/diagnosis): leptomeninges of the brain (1/ Ph^+ ALL), paraspinal soft tissues (1/AML), small intestine and the root of mesentery (1/AML), inguinal lymph nodes (1/AML), paranasal sinuses (1/AML). Treatment plans for those IER included (No. of cases/diagnosis): 1/involved-field radiotherapy (IF-RT) followed by chemotherapy (CHT) and interferon-alpha (2/ ALL), 2/ imatinib + CHT + steroids and methotrexate intrathecally (1/ ALL), 3/ imatinib + CHT (1/ ALL), 4/ CHT (2/ AML), 5/ dasatinib (1/ $CD117^+$ AML,), 6/ surgery (1/AML), 7/ surgery + IF-RT (1/CML). 7/9 patients died after a median time of 10 mts (range, 1 - 30) due to resistant systemic relapse and/or infectious complications, 2/9 are currently under CHT. *Conclusions.* Our data indicate that IER following alloHSCT occur predominantly in acute leukemia pts, being rarely observed in pts with CML. No cases of IER have been reported among CLL patients. Sites of IER vary widely among the pts with skin and/or subcutaneous tissue being frequently involved. Local radiation therapy seems to be effective treatment option, but it does not prevent from systemic relapse and should be followed by other therapeutic modalities. Our observations suggest also that insufficient graft *vs.* leukemia mechanism may result in unusual clinical appearance of disease progression, temporarily restricted to focal infiltrates that precede leukemic generalization. Occurrence of isolated extramedullary disease offers only a narrow window for quick intervention, however, optimal treatment remains a challenge.

Stem cell transplantation 3

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REVERSE SEROCONVERSION OF HEPATITIS B VIRUS (HBV) AFTER ALLOGENIC HEMATOPOIETIC STEM CELL TRANSPLANTATION IN HBV ENDEMIC AREA

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Background. Reverse seroconversion (RS) of Hepatitis B virus (HBV), appearance of HBsAg in patients previously negative for HBsAg, has been reported as complication after allogeneic transplantation. But the frequency of RS in literature is very wide range, varies from 14 % to 86%. *Aim.* Korea is endemic area for HBV infection. In this study, we evaluated the changes in HBV serology and examined the prevalence of HBV RS in single center in Korea. In addition, we attempt to evaluate how the HBV serologic status of recipients and donors before transplantation influence on changes in post-transplant HBV serologic status in recipients. *Material and methods.* A total of 288 patients underwent allogeneic hematopoietic stem cell transplantation (HSCT) between February 1996 and June 2008. We reviewed the medical records of 288 patients and examined changes in HBV serology. HBV serologic markers of donors were also evaluated. *Results.* HBsAg and anti-HBs were examined in all 288 patients prior to transplantation; 20 recipients were positive for HBsAg and 268 recipients were negative for HBsAg, and among 268 patients negative for HBsAg before transplantation, the proportion of anti-HBs positivity was 89.6 % (240 of 268 patients). Of the 268 patients, all but 12 patients were assessed for anti-HBc IgG before allo-HSCT; 150 patients (58.6%) had anti-HBc. Among 288 donors, 204 cases had information of HBsAg. Of these 204 donors, 199 were negative for HBsAg and 5 were positive for HBsAg. Among 199 donors without HBsAg, 163 cases were assessed for anti-HBc and 170 cases were assessed for anti-HBs; 57 donors (35.0%) were positive for anti-HBc and 108 donors (63.5%) had anti-HBs before transplantation. With a median follow-up period of 66.9 months (range, 11.6-160.2), 3 of 268 recipients negative for HBsAg before transplantation experienced RS (1.1 %). All of these 3 patients had anti-HBc and anti-HBs before transplantation. Among donors of these 3 recipients, 2 had information about HBV serolologic markers, and both of them were negative for HBsAg and anti-HBs. Of the 168 cases in which HBV serologic markers of both recipients and their donors were evaluated, 14 cases who were negative for anti-HBs prior to transplantation received stem cell from donors who had anti-HBs. Among 12 patients who had follow-up HBV serology, 1 of 4 patients who were in chronic HBV infection experienced HBsAg clearance after transplantation and 5 of 8 patients who had negative HBsAg and negative anti-HBs experienced acquisition of anti-HBs without vaccination. *Conclusion.* In this examination, the prevalence of RS among patients who were negative for HBsAg before transplantation was 1.1 %. This is very low in comparison to the results of other studies in non-endemic area for HBV infection. This might be related to high rate of positivity of anti-HBs in donors. In addition, transfer of anti-HBs from donors to recipients seemed to make adoptive immunity in some patients, which resulted in HBsAg clearance or acquisition of anti-HBs.

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LONG-TERM IMPACT OF IMATINIB ON THE OUTCOME OF ALLOGENEIC TRANSPLANTATION IN ADULT PHILADELPHIA-POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA: SIMILAR OUTCOMES BETWEEN REDUCED-INTENSITY AND MYELOABLATIVE CONDITIONING TRANSPLANTS

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Background. Imatinib in combination with conventional chemotherapy as a front-line treatment have demonstrated an improved complete remission rate and a better outcome in adults with Philadelphia-positive acute lymphoblastic leukemia (Ph-positive ALL). However, despite a short follow-up, most patients who do not undergo allogeneic stem cell transplantation (SCT) eventually relapse and die as a result of disease progression. On the other hand, high transplant-related mortality remains a major obstacle in a subset of transplants, particularly those with advanced age or concurrent co-morbid conditions. From this point of view, it is reasonable to look to reduced-intensity conditioning (RIC)-SCT as a way to provide a graft-versus-leukemia effect and a survival advantage for Ph-positive ALL patients who are poor candidates for myeloablative conditioning (MAC). *Aims.* Previously, we observed the positive impact of imatinib on the outcome of allogeneic SCT in adults with Ph-positive ALL (Blood 2005;105:3449). We report here the updated results of this study with a longer median follow-up of 48 months, and we focused particularly on the role of RIC-SCT. *Methods.* From 2000 to 2008, 92 consecutive adults with newly diagnosed Ph-positive ALL who received allogeneic SCT following imatinib therapy were enrolled in this study. Donor grafts were matched sibling donor (n=55), well-matched unrelated donor (n=15), partially matched unrelated donor (n=12), and mismatched unrelated donor (n=10). Of the whole group, 83 (90.2%) patients were transplanted in CR1. Sixty-nine (75.0%) patients received MAC (total body irradiation-based), and the remaining 23 (25.0%) patients received RIC [fludarabine (150 mg/m^2) plus melphalan (140 mg/m^2)]. The indications for RIC-SCT were: (1) aged more than 50 years (n=7) and (2) decreased organ function or active infections (n=16). All patients received the same graft-versus-host disease prophylaxis consisting of calcineurin inhibitor (cyclosporine for sibling transplants; tacrolimus for unrelated transplants) plus methotrexate. All patients and donors provided written informed consent, and the treatment protocol was approved by the institutional review board of The Catholic University of Korea. *Results.* For all transplants, median age was 35 years (range, 16-59 years). The incidence of acute (grade II-IV) and chronic graft-versus-host disease were 57.3% and 55.3%, respectively. The 4-year cumulative incidence of relapse and non-relapse mortality were 21.1% and 19.3%, respectively, and the 4-year disease-free survival and overall survival rates were 63.1% and 64.4%, respectively. Although RIC transplants were older (45 years *vs.* 30 years, P<0.001) and received peripheral blood stem cells more frequently (91% *vs.* 22%; P<0.001) as compared to MAC transplants, overall transplantation outcomes were not significantly different in terms of relapse (15.2% *vs.* 22.9%, P=0.526), non-relapse mortality (30.8% *vs.* 15.5%, P=0.301), disease-free survival (58.7% *vs.* 64.5%, P=0.834), and overall survival (57.8% *vs.* 66.6%, P=0.687). *Summary/Conclusions.* Our long-term follow-up data suggest that the positive impact of imatinib therapy on the outcome of allogeneic SCT is well preserved. In addition, RIC-SCT is a potential therapeutic approach for adults with Ph-positive ALL who are not eligible for MAC-SCT. Large prospective studies are needed to elucidate the role of RIC-SCT for adult Ph-positive ALL in the era of tyrosine kinase inhibitors.

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IN VITRO DIFFRENTIATION POTENTIAL OF MESENCHYMAL STEM CELLS DERIVED FROM NATAL, WISDOM, DECIDUOUS TEETH DENTAL PULP AND HUMAN BONE MARROW: A COMPERATIVE STUDY

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Background. Their characteristics of high proliferation, self renewal capacity even in later passages, multi-lineage differentiation and their simplicity in the isolation make mesenchymal stem cells (MSCs) considerably important sources for regenerative medicine. Considerably, MSCs obtained from patients and donors can be used in many applications of tissue engineering, in which the obtaining of tissue in most suitable cell/tissue culture conditions by growing the isolated cells on biocompatible/biodegradable scaffold. In particular MSCs derived from different sources were usually used experimentally in the improvement of bone defects and in the development of tissue grafts. *Aim.* It is important to know osteogenic differentiation capacity of MSCs derived from different sources in detail for finding appropriate source in future studies and applications. Because of this reason in our study we used human bone marrow (hBM)(n=3), human exfoliated deciduous tooth dental pulp (hDP)(n=2), human natal tooth dental pulp (hNDP) (n=2) and human wisdom tooth dental pulp (hWDP) (n=2) derived mesenchymal stem cells to determine osteogenic differentiation capacity. *Methods.* All cells were treated with osteogenic stimulatory medium for 30 days and the alkaline phosphatase activity (ALP) assay were performed on the cells at 3., 7., 11., 14. and 21. days after the begining of stimulus. Absorbance was read at 405 nm and ALP activity was normalized by total protein concentration. In addition, at the end of 4 weeks osteogenic differentiation was assessed by staining with Alizarin Red,

Osteocalcin, Osteonectin, BMP2 and BMP4. *Results.* In all groups, cells were displayed positive reaction for osteogenic markers (Osteocalcin, Osteonectin, BMP2 and BMP4), and bone nodules stained with Alizarin Red S were observed. The results demonstrate that in the 3. day from the begining, the ALP activity started to increase and at day 14, activity was highest, and in later days, it decreased in all groups. We determined that hBM MSCs displayes highest capacity for osteogenic differentiation. However, at 7.,11. and 21. days, second highest ALP activity was observed in hNDP-MSCs. *Conclusions.* We demonstrated here that hBM MSCs and hNDP MSCs were most efficient sources for bone tissue engineering researches and their applications. In addition, hDP and hWDP derived mesenchymal stem cells can be use for bone regeneration studies as an alternative source for the reason that these tissues are obtained easy from patients or donors. Although hNDP can be obtained rarely as a potential source of mesenchymal stem cell for tissue engineering applications, they can be used in experimental approaches.

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A COMPARISON OF FUNCTIONAL AND PHENOTYPIC FEATURES OF MOBILIZED CD34⁺ CELLS IN PATIENTS AND HEALTHY DONORS BASED ON THE INTENSITY OF THEIR RESPONSE TO G-CSF.

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Background. Since the mid 1985 the combination of hematopoietic growth factors and/or chemotherapy is applied to allow mobilization of CD34⁺ cells from marrow to the blood. Once harvested, this cell population, containing both progenitors and stem cells (HPC/HSC), has become the common source for autologous or/and allogeneic transplantations procedures. *Aims.* Indeed, the new development of cell-based therapies in hematologic settings requires the understanding of mechanism involved in HPC/HSC migration and cell cycle kinetics during mobilisation. With respect to the percentage and absolute number of CD34+ cells after Granulocyte-Colony Stimulating Factor mobilisation (G-CSF) the patients (Pts) and healthy donors were divided in two groups: Good mobilizers (GM) and poor mobilizers (PM). *Methods.* We studied in their CD34⁺ cells: cell cycle (Ki67/IP staining), clonogenic and *ex vivo* expansion potential, aldehyde deshydrogenase activity (Aldefluor), expression of some adhesion molecules relevant for CD34⁺ cells mobilisation as: VLA-4, LFA-1 and CXCR4. *Results.* Twenty Two Pts (Myeloma=13 and Lymphoma=9) and 9 healthy donors underwent G-CSF mobilisation with or without chemotherapy. CD34⁺ cells were purified using the MACS cell isolation kit and Mini-Macs columns obtaining a purity of more than 88%. Based on the percentage of circulating CD34⁺, 2 populations of subjects were identified (<0.1%: PM and >0.1%: GM). Total white blood cells number was higher in PM arm (48.8 G/l) than in GM arm (36.75) (P=0.03). The median pCD34+ cell was higher in GM (60:31-153) than in PM (35:11-64) (P=0.001). Interestingly, PM showed more G0 cells than GM with mean percentages 46±10 and 31±10 respectively (P=0.004). Indeed, the percentage of the S/G2/M fraction of CD34⁺ cells was higher in GM (2±0.9) than in PM (0.69±0.7) (P=0.002). Moreover, there was a trend to observe a higher total nucleated cells fold expansion potential in PM arm 27±11 *vs.* 17±13 in GM (P=0.06). It should be stressed that those results were observed also when Pts and healthy donors were studied separately. Analysis of ALDH activity revealed an unexpected population of CD34⁺ cells that were ALDH⁺ despite their CD133 negativity. These CD34⁺ALDH⁺CD133⁻ cells were present in both Ps and healthy donors but were more pronounced in Pts (38% in PM *vs.* 9% in GM; P=0.01). Moreover, GM among healthy donors showed more cells expressing VLA-4 (87% *vs.* 61% in PM arm, P= 0.05). The analysis of the data related to neutrophils and platelets recovery after autologous HSC transplantation and available for 16 patients among 22 showed a reduced median duration of neutropenia $<0.5\times10^9$/L in PM (Pts= 6) in comparison to GM (Pts=10) respectively 9 days (7-24) for GM and 7 days (5-8) for PM (P=0.02); while the median duration of thrombopenia $<20\times10^9$/L was: 7 days (4-14) in GM and 4 days (3-5) in PM group. These observations are important since no statistically significant difference was observed in the median number of CD34⁺ cells infused between GM (median 2.42; range 1.5-6.9) and PM (median 3.04; range 1.9-3.9) in these patients not heavily pretreated (1 line=11 Pts, 2 lines=5 Pts). *Conclusion.* These results suggest important phenotypic and functional variations in CD34⁺ cells between PM and GM (cell cycle, expression of CD133 by ALDH⁺ cells) as well as some differences between the healthy donors and Pts (VLA-4 expression). These differences are confirmed as for the early neutrophils and platelet engraftment after myeloablative chemotherapy. Further studies will be required on a larger cohort of Pts and healthy donors in the context of rapid and durable recovery of hematopoietic function.

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THE IMPACT OF MINOR HISTOCOMPATIBILITY ANTIGENS MISMATCH ON SIBLING CORD BLOOD TRANSPLANTATION FOR THALASSEMIA

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Cord blood (CB) transplantation from HLA-identical siblings is becoming an effective treatment for thalassemic patients, for whom HLA compatibility represents a stringent requirement. Besides major histocompatibility complex (MHC) antigens, minor histocompatibility antigens (mHAgs) may be considered to play a role in this setting. In HLA identical sibling transplantation, mHAgs mismatches can lead to T-cell activation with specific alloreactivity against recipient's or donor's antigens, thus supporting the Graft-Versus-Host Disease (GVHD) or rejection response respectively. To investigate the role of mHAgs, 9 thalassemic patients and their CB sibling donors were typed for 12 mHAg : HA-1, HA-2, HA-3, HA-8, HB-1, ACC-1, ACC-2, CD31 (codons 125, 563 and 670), CD62L (codons 206 and 213), PANE-1, UGT2B17, SP110 (mHA Minitray kit, University Clinic Heidelberg, Germany). The characteristics of CB units are shown in the table. The patients were omogeneous for age, weight, diagnosis, conditioning regimen and GVHD prophylaxis. Moreover each donor/recipient pair was HLA identical (two digits for the class I and four digits for the class II). The examined group represented an ideal model thanks to the characteristics of the patients, that are well standardized allowing to exclude other variable factors related to donor (HLA and cell dose) and transplant (conditioning and GVHD prophylaxis regimen). We studied the mHAgs-mismatching grade in both GVHD and rejection directions. As shown in the graph, the greater was the number of donor *vs.* recipient mismatches the shorter was the neutrophil and platelet recovery time; on the contrary, the recovery time increased considering the recipient *vs.* donor disparities. All patients are alive and well, no-one developed acute or chronic GVHD. Moreover no-one experienced graft failure, all recipients reaching full-donor chimerism, except of two (10% recipient residue) and one (20% recipient residue).

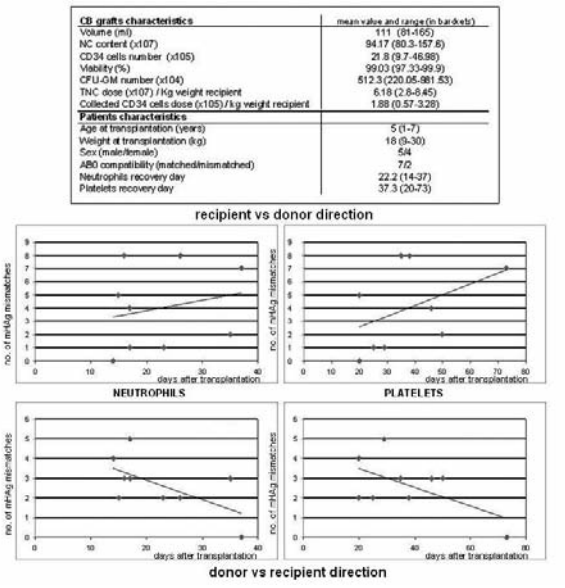

Figure.

The lower impact of donor *vs.* recipient mHAgs mismatches on the recovery times confirmed the natural tolerogenicity of the CB graft. All the more so because for thalassemia patients no Graft-Versus-Leukemia

(GVL) effect is required and even a light GVHD risk is not accettable. On the contrary, recipient *vs.* donor mHAg mismatches correlated with delayed engraftment of neutrophils and platelets. The analysis of these nine patients showed that the the recipient presenting the lower recovery time (+14 days for neutrophils and +20 days for platelets) had no recipient *vs.* dionor mismatches. Consequently recipient's T-cells recognizing mismatched mHAgs of the cord could be responsible for the delayed recovery. Anyway the disparities analyzed seemed not to translate into a concrete increased risk of graft failure, as the observed recovery times remained under a minimum limit and all patients engrafted. Further investigations are needed to confirm the role of mHAgs by enrolling in the study an increased number of patients and performing a qualitative analysis of mHAgs, in particular the most immunogenic phenotypes.

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LONG-TERM IMMUNE RECONSTITUTION FOLLOWING STEM CELL TRANSPLANTATION IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Chronic lymphocytic leukemia (CLL) is associated with profound immune disturbances, which are exacerbated by therapy. Although CLL is still considered incurable, long term remissions are observed in patients after allogeneic stem cell transplantation (SCT). The extent of recovery of immune function in such patients remains unknown *Aim.* To investigate the immune function of 21 patients with CLL who were in long complete remission following autologous (n=6) and allogeneic (n=15) SCT. *Methods.* Lymphocyte subsets were studied using multiparameter flow cytometry and compared to healthy controls. Immunoglobulin subtypes, complement proteins, and β2-microglobulin (B2M) were quantified by standard techniques and IL-10 and VEGF by flow-based cytometric bead array technology. CD8+ T cell response to CMV was assessed using a pentameric HLA-A2 binding CMV pp65-derived peptide and functionality was confirmed using an interferon gamma (IFNγ) ELISPOT. *Results.* Median age was 51 (range, 33-64) and median follow-up since SCT was 6 years (range, 2-17). Three patients had chronic graft-versus host disease (cGVHD) at sample collection, with two receiving immunosuppression. Three patients (all post allogeneic SCT) had detectable residual CLL cells ($>10^{-4}$) in peripheral blood despite being in CR. In the remaining 18 patients with no detectable minimal residual disease (MRD), normal $CD19^+CD5^-$ and $CD19^+CD5^+$ B cell populations were higher than in healthy individuals (160/mm^3 *vs.* 88/mm^3; P=0.008 and 24/mm^3 *vs.* 1/mm^3; P=0.003 respectively). Although CD4 and CD8 counts were similar in patients and controls, an abnormal CD4:CD8 ratio was seen in 8 out of 21 patients and 4 patients had a $CD4^+$ T cell count <400/mm^3. In contrast, $CD8^+$ T cells with a chronically activated phenotype, $CD3^+CD8^+DR^+$, were significantly elevated compared to controls (200/mm^3 *vs.* 78/mm^3; P=0.05). The nine CMV^+/HLA-A2 patients all showed specific cytotoxic $CD8^+$ T cells which exhibit predominantly a $CD45RA^+CD27^-$ phenotype. An expansion of $CD45RA^+CD27^-$ cells was only observed in CMV positive patients. The ability of these cytotoxic T cells to secrete IFNγ in response to pp65 was confirmed by ELISPOT. All patients showed a functional response to a peptide pool containing a mixture of CMV, EBV and influenza peptides. $CD4^+CD25^+FOXP3^+$ regulatory T cells were significantly elevated in CLL patients compared to controls (3.6% *vs.* 1.95% of $CD4^+$ cells; P=0.03). There were no quantitative abnormalities in $CD3^-CD56^+$ cells. Prior to transplant, hypogammaglobulinemia was present in most of the patients. Fourteen patients restored their serum levels of immunoglobulin after SCT whereas 7 patients persisted with hypogammaglobulinemia. Three of these were in CR but MRD positive, and two patients were receiving immunosuppression for cGVHD. Only two patients had otherwise unexplained persistent hypogammaglobulinemia. Complement proteins C3 and C4 were all within the normal range. The direct Coombs test (DCT) was also negative in all patients. B2M levels were increased (>2.5 mg/dL) in 5 out of 21 patients. No significant differences were found in IL-10 and VEGF levels between patients and normal controls. *Conclusion.* These data suggest that immune function is not completely restored in CLL patients with a long CR post-SCT, even in those who are MRD negative.

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ASSESSMENT OF IRON OVERLOAD WITH T2*MRI IN EX-THALASSAEMIC PATIENTS AFTER STEM CELL TRANSPLANTATION: AN INTRIM REPORT

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Background. The excess iron acquired during years of thalassaemia eliminate slowly after hematopoetic stem cell transplantation (HSCT). T2* relaxation time approach could be used as an appropriate and non invasive method in the assessment of myocardial and liver iron overloaded after HSCT. *Aims.* To assess the changes of myocardial and liver iron concentration after successful HSCT by T2*MRI. *Methods.* Fourteen beta-thalassaemic patients were assessed for tissue iron overload by serum ferritin level, liver biopsy and T2* MRI of heart and liver before HSCT and at the end of sixth month of ex-thalassaemia feature. Chelation was not started in this time. *Results.* Myocardial T2* (mean 23.47ms ±10.91 befor, *vs.* 20.03 ms±9.77 SD, after) and liver iron concentration estimated by MRI (mean 5.23mg/g±3.33 befor, *vs.* 5.34 mg/g±3.13 SD, after) and liver iron score were not changed significantly after 6 months ex-thalassaemia feature. There was no significant correlation between myocardial T2* values and liver iron concentrations either before or after HSCT. Although serum ferritin levels and liver T2* 6 months after HSCT were correlate significantly (P<0.001), it was not true for myocardial values. *Conclusions.* T2*MRI allows monitoring of iron deposition in a non invasive way after HSCT and could be even used instead of liver biopsy before HSCT in beta-thalassaemia patients. Serum ferritin is an inaccurate parameter to guide iron chelation after transplantation but T2* values before HSCT are usually enough for early treatment.

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A SHORT BORTEZOMIB-COURSE RESTORES THE BLUNTED IMMUNOMODULATORY PROPERTIES OF MESENCHYMAL STEM CELLS IN AN INFLAMMATORY MILIEU OF EXPERIMENTAL ARTHRITIS, PROVIDING SIGNIFICANT CLINICAL BENEFIT

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Background. Mesenchymal stem cells (MSCs) exert unique immunomodulatory properties and currently a lot of research has been dedicated to the investigation of MSCs in autoimmune diseases. However, their potential in treating rheumatoid arthritis in animal models has been thus far contradictory. The inflammatory milieu in collagen-induced arthritis (CIA) has been proposed to inhibit their immunosuppressive effects preventing thereby their speculated therapeutic benefit (Djouad *et al.*, Arthr Rheum 2005). In other reports, infused MSCs were shown to ameliorate CIA (Augello *et al.*, Arthr Rheum 2007/Gonzalez *et al.*, Arthr Rheum 2009). We have shown (under revision) that Bortezomib (Bzb), a potent NF-kb inhibitor, significantly ameliorates advanced adjuvant-induced arthritis (AA) by reducing inflammation and bone loss. *Aims.* We sought to investigate whether MSCs could play a therapeutic role in a rat model of AA. Given the strong anti-inflammatory capacity of Bzb, we also explored the effect of a short Bzb-course, aiming to alter the inflammatory AA environment before MSCs infusion. *Methods.* Bone marrow-derived MSCs were identified by FCM analysis and differentiation assays for trilineage potential. Four groups of animals were tested: AA control, MSCs-alone, Bzb-alone and Bzb+MSCs. Bzb (0.25 mg/kg, ip) was initiated at the onset of arthritis (day 13,16,19 after AA-induction) and 8-14×10^6/recipient allogeneic (allo) MSCs were infused ip during advanced disease (day 21, 25 after AA-induction). Rats were sacrificed and target tissues excised for analysis on d32. The proliferation of AA-Fibroblast-Like Cells (FLS) and Con-A activated AA-splenocytes (SPLCs), cultured in the presence or absence of MSCs or MSC supernatant (sup), was measured by thymidine incorporation. $CD4^+/CD25^+/Foxp3^+$ (T-reg) cells in blood were determined by FCM. A pattern of pro- and anti-inflammatory cytokines in serum and in the sup of cultured SPLCs was measured by a cytometric bead array. *Results.* AA-FLS or Con-A activated AA-SPLCs cultured in the presence or not of MSCs sup, were inhibited in a dose-dependent manner (P=0.02 P=0.018, respectively). Cell to cell contact of MSCs with AA-SPLCs -even at a minimal ratio of MSCs/SPLCs 0.05/1- resulted in profound inhibition of SPLCs proliferation(P=0.000004). Despite

the strong inhibition *in vitro* of AA-target cells by MSCs, AA-rats treated with MSCs-alone didn't show signs of improvement compared with control rats. In contrast, Bzb+MSCs-treated animals demonstrated significantly decreased arthritis score over AA-, MSCs-alone- Bzb-alone-treated animals (4.3±0.8 *vs.* 9.3±2.2 *vs.* 10.3±2.0 *vs.* 7.5±2.5/P=0.005, P=0.009, P=0.03, respectively). The clinical remission was in line with markedly improved joint histology. After Bzb and before MSCs infusion (day21) a significant increase of blood T-regs and restoration of SPLCs proliferation was observed in treated animals and both were further improved after MSCs infusion. An altered cytokine secretion pattern (decreased levels of IL-6, TNF-a) was detected in serum of Bzb-treated rats before the infusion of MSCs. After infusion of MSCs, increased IL-10 and decreased IFN-γ were counted in the sup of SPLCs from Bzb+MSCs-treated rats. *Conclusions.* MSCs exert clinical benefit in AA only in combination with Bzb. Early administration of a short Bzb-course allows MSCs to retain their immunomodulatory function by probably altering the inflammatory AA milieu.

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THE COMBINATION OF AMD3100 + G-CSF SUCCESSFULLY MOBILIZES HSCS INTO THE PERIPHERAL BLOOD COMPARED TO G-CSF ALONE, IN A THALASSEMIC MOUSE MODEL

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Gene therapy (GT) for thalassemia has been recently brought to the clinic and G-CSF-mobilized hematopoietic stem cells (HSCs) may represent the optimal source of autologous cells for genetic modification due to the higher yield of $CD34^+$cells compared to bone marrow (bm) harvest. We have previously shown that G-CSF-mobilization in thalassemic (HBBth-3) mice is less efficient than normal (C57Bl6) mice due to increased trapping of hematopoietic stem (Lin-sca-1+ckit+/LSK) and progenitor (CFU-GM) cells in the enlarged thalassemic spleen. AMD3100, a reversible inhibitor of the CXCR4/SDF-1 interaction, has been shown to induce rapid mobilization of $CD34^+$ cells in healthy donors and cancer patients. In the current study we explored whether AMD alone or in combination with G-CSF could improve the mobilization efficiency of thalassemic mice. C57Bl6 and HBBth-3 mice received G-CSF-alone (250 μg/kg×7days), AMD-alone (5 mg/kg×3days) or the combination of two. Hematopoietic tissues (blood, bm, spleen) were collected and the LSK and CFU-GM were assayed (by FCM and clonogenic assays) and calculated in absolute numbers. Spleen weight was determined as a ratio (×1000) to body weight. For migration assays, bm cells were cultivated in transwell system in the presence or not of the chemoattractant SDF-1. AMD-alone didn't significantly improve the HSC yield in thalassemic mice as compared to G-CSF mobilization (LSK/μL blood:103±85 *vs.* 67±19, P=ns). In contrast, the combination of AMD3100+G-CSF restored the less efficient mobilization (LSK cells/μL blood: 224±104 *vs.* 67±19 P=0.04, CFU-GM/mL blood: 1671±984 *vs.* 330±123 P=0,05, respectively) and resulted in successful mobilization at levels comparable with normal G-CSF-mobilized mice (LSK cells/μL blood:224±104 *vs.* 197±162 P=ns, CFU-GM/mL blood:1671±984 *vs.* 1429±1020 P=ns, respectively). AMD released stem cells to the circulation by detaching them from bm because reduced numbers of bm LSK cells were counted in the AMD-alone as compared to the untreated group (LSK/2 femurs×10^3: 692±429 *vs.* 1472±1049, P=0,05). In contrast, G-CSF mobilized HSCs by inducing bm hyperplasia before peripheralization of HSCs (LSK/2femurs×10^3: 2684±1743 *vs.* 1687±1016 P=0.02 / CFU-GM/2femurs:111841±15391 *vs.* 76774±31728 P=0.01). In contrast also to G-CSF, AMD-alone did not cause increased trapping of stem and progenitor cells in the spleen and importantly, it rather forced the egress of HSCs from the spleen (LSK cells/spleen×10^3, *vs.* the untreated condition: 5719±2999 *vs.* 8303±4515 P=0.1). Consequently, the addition of G-CSF to AMD did't not induce marrow hyperplasia (LSK/2femursX103 *vs.* untreated: 1681±862 *vs.* 1472±1049, P=ns/ CFU-GM/2femursX102: 863±262 *vs.* 887±317, P=ns) or increased splenic accumulation of HSCs (LSK cells/spleen×10^3: 8415±3718 *vs.* 8303±4505 P=ns) compared with the steady-state condition. Although AMD-alone didn't significantly increase the spleen size in thalassemic mice [spleen ratio (vs untreated): 26±4,1 *vs.* 23±3.3, P=ns], the combination of AMD3100+G-CSF still resulted in significant spleen size increase over the untreated condition (31.6±8,6 *vs.* 23±3.3, P=0,02). Bm cells of the AMD+G-CSF group demonstrated increased migration to SDF-1 compared with AMD-alone, G-CSF-alone and steady-state condition (57% *vs.* 17% *vs.* 28% *vs.* 18.4%) implying a better engraftment profile after transplantation. AMD3100+G-CSF may be proved beneficial for obtaining high numbers of HSCs with enhanced engraftment potential in a thalassemia GT setting.

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OLDER AGE, THE NUMBER OF PRECEDED EVENTS OF ACUTE KIDNEY INJURY AND THE IN VIVO T CELL DEPLETION ARE RISK FACTORS FOR KIDNEY INJURY AFTER HEMATOPOIETIC CELL TRANSPLANTATION

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Background. Chronic kidney disease (CKD) is one of the late effects following allogeneic stem cell transplantation (HCT) and has been associated with a variety of factors. *Aim.* Evaluation of Kidney injury after hematopoietic cell transplantation in terms of incidence, risk factors and outcome. *Methods.* We retrospectively evaluated the development of CKD in 164 patients (pts), aged 9-65 years, after a median follow up of 7 (3-120) months. The patients underwent HCT for hematological disease from sibling (137) or unrelated donors (27) after myeloablative (144) or reduced intensity conditioning regimens (20) and survived for at least 3 months post transplantation (median follow up 23.5 months). CDK was defined as a GFR<60 mL/min/1.73 m^2, estimated by the modification of diet in renal disease (MDRD) formula and the Schwartz formula for children. *Results.* The median time for CKD development was 6 months with a probability of 23.8% at 18 months. The mean value of preHCT- GFR was within normal limits (111.5±26) for pts who did not develop CKD and 97.21±19 for those who developed CKD, while the GFR at 12 months post transplant was 108±28 and 54.7±5.4 (ml/min/1.73 m^2), respectively. The course of CKD was asymptomatic until the end-stage disease when 3 pts were on dialysis and 1 patient received a renal transplant from his mother. On univariate analyses, the probability of developing CKD was 25% at 18 months for pts with 0 or 1 event of kidney injury *vs.* 60% for those with 2-5 preceded events (P:0.006). The type of conditioning, hyper fractionated TBI, administration of Thiotepa or Fludarabine, acute or chronic GVHD, the toxicity of antiviral or antifungal treatment did not correlate with the CKD. Calcineurin inhibitors were not included as risk factors because of the universal administration as prophylaxis and because of their toxicity appearing in the long term treatment. On multivariate analysis, the only predictive factors were older age (P=0.01), the number of preceded events of acute kidney injury, and the *in vivo* T cell depletion with antithymocyte globulin or alemtuzumab (0.013). The CKD did not influence the non relapse mortality of the HCT. Summary/*Conclusions.* Kidney injury seems to be an early complication after hematopoietic cell transplantation because the number of preceded events of acute kidney injury, *in vivo* T cell depletion and older age were found to be the main risk factors in multivariate analysis. The profound lymphocytopenia of T-cell depletion might possibly be related to an excess of secretion of the transforming growth factor beta (TGF beta) which may promote early and rapid renal fibrogenesis. Close monitoring of the patients for early events of acute kidney injury due to drug toxicity is necessary for CKD prevention.

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FACTORS AFFECTING THE OUTCOME OF PLERIXAFOR-BASED HEMATOPOIETIC STEM CELL MOBILIZATION

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Plerixafor is a CXCR4 receptor inhibitor which was recently introduced for stem cell mobilization in myeloma and lymphoma patients

identified as proven or predicted poor mobilizers. In order to assess the efficiency and factors affecting the outcome of plerixafor-based stem cell mobilization, we retrospectively collected data from 11 Polish centers that used this drug. In total, data regarding 57 patients was collected. 21 of them (37%) suffered from multiple myeloma, 17 of non-Hodgkin's lymphoma (30%) and 19 of Hodgkin's lymphoma (33%). The ratio of males to females was 32/25 and the median age was 51 (range 19-71). All of them have been treated with chemotherapy with the median of 12 courses (range 4-37). 14 patients (25%) have been treated with radiotherapy and 10 (18%) underwent autologous hematopoietic stem cell transplantation in the past. 48 patients (84%) failed at least one previous mobilization attempt, while in 9 (16%) plerixafor was used in first-line mobilization. At the time of mobilization with plerixafor, 24 (42%) were in complete remission, 25 (44%) in partial remission, 5 with stable disease (9%) and 3 (5%) with progressive disease. In 9 patients (16%) plerixafor was added to chemotherapy-based mobilization regimen. Based on available data, median number of 22 circulating $CD34^+$ cells/μL (range, 0-121) was observed following first plerixafor administration and in 28 patients (60%) it exceeded the No. of 20 $CD34^+$ cells/μL. In 48 patients (84%) median of 2 leukapheresis were performed (range 0-4) and the median No. of $2,8\times10^6$ $CD34^+$ cells/kg b.w. was collected (range 0,17-8,0). The total dose of $> 2,0\times10^6$ $CD34^+$ cells/kg b.w. required for autologous stem cell transplantation was collected in 38 patients (67%). The median No. of nucleated cells in stem cell product was 10.2×10^8/kg b.w. (range 1.3-40.4) and the median volume was 650 mL (range 169-2050). The univariate analysis (Chi-Square Test) revealed that diagnosis of non-Hodgkin's lymphoma, previous radiotherapy and mobilization line >2 were associated with significantly lower probability of collection of sufficient No. of $CD34^+$ cells. Only 41% of patients with non-Hodgkin's lymphoma *vs.* 76% of myeloma and 79% of Hodgkin's lymphoma patients have been efficiently mobilized ($P<0.05$). Similarly, only 43% of patients after radiotherapy collected required No. of $CD34^+$ cells compared with 74% of patients not treated with radiotherapy ($P<0.05$). All patients who received plerixafor in first mobilization attempt collected required No. of stem cells, contrary to 60% of those who failed at least one previous mobilization ($P<0.05$). This has been confirmed in multivariate logistic regression analysis and age adjusted relative risk for collection of sufficient number of $CD34^+$ cells was higher for patients without previous radiotherapy and lower for NHL patients (Odds Ratio 5.,74 and 0.09, correspondingly). In summary, plerixafor-based mobilization regimen was proven successful in majority of patients and it seems to be affected by similar risk factors as traditional mobilization regimens. However, in order to make final conclusions, collection of data from larger number of patients is warranted.

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GRAFT-VERSUS-TUMOR EFFECT OF CHRONIC GRAFT-VERSUS-HOST DISEASE IN THE SETTING OF UNRELATED TRANSPLANTATION

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Background. Recently, there has been a marked increase in the number of unrelated hematopoietic stem cell transplantation (URD-SCT). The use of peripheral blood stem cells (PBSCs) has been rapidly increased in the setting of URD-SCT, which is caused by easy acquisition and donor choice, and previous reports demonstrating more rapid engraftment, similar survival outcomes and graft-versus-host disease (GVHD) rates compared to bone marrow (BM) stem cells. Recent some reports also raised concerns of late chronic GVHD-related morbidity and mortality. However, there has been few data about the presence of graft-versus-tumor (GVT) effect in the setting of URD-SCT. *Aims and methods.* To investigate the effect of pre-transplantation factors such as stem cell source, infused cell numbers, conditioning intensity, and the kind of donor registries on key outcomes and the presence of GVT effect, we retrospectively analyzed 303 patients who underwent URD-SCT from January 2003 to December 2008 for hematologic diseases in Catholic Blood and Marrow Transplantation Center. *Results.* HLA matching was based on molecular typing for HLA-A, -B, -C and DRB1, and well-matched cases, partially mismatched cases, and mismatched cases were 46%, 40%, and 14%, respectively. Donors were found in two Korean registries (78%) and four foreign registries (22%). Stem cell source consisted of BM (60%) and PBSCs (40%), and disease status at transplantation were standard (81%) and advanced (19%). The intensities of conditioning regimens were myeloablative (MAC, 76%) and reduced-intensity conditioning (RIC, 24%). With a median follow-up of 33 months (range, 3-76), 3-year overall survival (OS), disease-free survival (DFS), and cumulative incidences of relapse (RI) and non-relapse mortality (NRM) were 58%, 51%, 26% and 24%, respectively. The cumulative incidences of acute GVHD grade II-IV and chronic GVHD were 53% and 59%. On multivariate analyses, both mismatched HLA and advanced disease status at transplantation were major independent factors predicting lower OS and DFS, and higher NRM. Additionally, NRM was significantly higher in patients transplanted from four foreign registries compared to two Korean registries, but they included significantly higher number of donors with mismatched HLA and recipients with malignant diseases or advanced disease status at transplantation. The use of PBSCs was an independent factor associated with the development of both acute and chronic GVHD. On the other hand, there were no differences of key outcomes according to stem cell sources (BM *vs.* PBSCs), conditioning intensity (MAC *vs.* RIC), and infused cell numbers of $CD34^+$ or $CD3^+$ cells in both all patients and patients who received PBSCs. Notably, in patients with malignant diseases, we found that patients who developed chronic GVHD had significantly higher OS and DFS, and lower RI on multivariate analyses adjusted with significant pre-transplantation factors affecting each outcome, which reflects the presence of GVT effect in the setting of URD-SCT. *Conclusion.* These single center data demonstrated the GVT effect of chronic GVHD in the setting of URD-SCT, in contrast to CIBMTR data (Biology of Blood and Marrow Transplantation 2007:13;1461-8). It should be further investigated with large prospective studies of homogeneous cohort.

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IRON OVERLOAD IN HEMATOPOIETIC STEM CELL TRANSPLANT (HSCT) RECIPIENTS

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Background. transfusional iron overload (IO) can cause severe long term morbidity among HSCT recipients. Objective: to analyze the iron status of multitransfused HSCT pts and the effectiveness of iron depletion therapy of those with IO. Patients and method: during the period Sept'04 - Sept'09, 213 HSCT were performed in our institution. Since the majority of the pts had been referred for HSCT from different centers and were discharged from our institution by day +100, we do not have a proper follow-up of a good proportion of them. Long-term surviving pts who continued their follow-up at our Center were screened with serum iron studies after day +180. To be considered evaluable, high ferritin levels had to be confirmed at least twice at a minimum interval of one month, and any concomitant acute process had to be ruled out.

Table .

Type of depletion	Pts	PRBC Transfused	Ferritin (ng/ml) pre-depletion	Ferritin (ng/ml) post-depletion	Phlebotomies to ferritin <1000 ng/ml	Months to ferritin <1000 ng/ml
Phlebotomies	9	14-55*	1039-6647*	217-925*	3-27*	3-28*
Deferasirox	5	26-70*	1289-2673*	362-494*	-	1-2*
Both	1	26	3047	395	8	9
*Range						

Results. fifty-nine pts were fully evaluable (54% male, 46% female). Median age was 53 years (range: 9-68). The pts were diagnosed with: 16 MM (27%), 11 NHL (19%), 8 AML (13%), 6 ALL (10%), 6 HL (10%), 4 MDS (7%), 3 CLL (5%), 3 AA (5%), 1 CML (2%), and 1 WM (2%). Twenty-nine pts (49%) had undergone allo-HSCT (21 from matched sibling donors, 8 from unrelated donors) and 30 (51%) auto-HSCT. Median follow up was 12 months (6-45). The patients received 1 to 70 PRBC units. Eighteen pts were transfused with more than 20 PRBC and 41 pts got less than 20 PRBC. Among the first group of pts, 89% (16/18) reached ferritin levels > 1000 ng/mL (median: 1742; range: 1025-6647). Among the second group of pts, just 7% (3/41) reached ferritin levels

> 1000 ng/mL; the median ferritin of the resting 38 pts was 316 ng/mL (range: 67-872). No pts transfused with less than 13 PRBC had ferritin levels > 1000 ng/mL. Fifteen (79%) out of the 19 pts with ferritin levels > 1000 ng/mL underwent some kind iron depletion therapy: 9 with repeated phlebotomies (all allo-HSCT), 5 with deferasirox (4 allo-HSCT, 1 auto-HSCT) and 1 pts with both (allo-HSCT) (see table); the remaining 4 pts with ferritin >1000 ng/mL (6 to 9 months after auto-HSCT) are currently been evaluated for depletion. Allo-HSCT pts who received deferasirox were included in a multicenter clinical trial. (Introduced Table). *Conclusions.* 1. A good correlation was found between the number of PRBC units transfused and the serum ferritin six months post-HSCT, measured under appropriate conditions. 2. Phlebotomies and deferasirox were effective for iron depletion. 3. Deferasirox was well tolerated and faster than the therapeutic program of phlebotomies for reducing IO in the post-HSCT setting.

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ROLE OF IMMUNOSUPPRESSIVE TOTAL NODAL IRRADIATION-BASED RECONDITIONING REGIMENS AFTER GRAFT REJECTION OR GRAFT FAILURE IN PEDIATRIC PATIENTS TREATED WITH MYELOABLATIVE ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. Primary non-engraftment or early graft rejection after myeloablative allogeneic hematopoietic stem cell transplantation (=HSCT) represent a life-threatening complication because patients are often in poor clinical condition and at high risk for fatal infections increasing with time of pancytopenia. Since graft failure is a rare event, standardized treatment protocols addressing the type of non-myeloablative reconditioning regimens prior to a second transplantation are lacking. *Aims.* The retrospective analysis aims to address the efficacy of total nodal irradiation (TNI)- based reconditioning regimens on pediatric patients with graft failure or graft rejection after allogeneic HSCT. *Methods.* The results of 14 pediatric patients with different hematologic diseases (ALL (n=5), MDS (n=4), CML (n=2), AML (n=1), paroxysmal nocturnal hemoglobinuria (n=1), Kostmann disease (n=1)) treated with a TNI-based reconditioning regimen were retrospectively analysed. TNI consists of a supradiaphragmatic "mantle" field encompassing the cervical, supraclavicular, infraclavicular, axillary, hilar and mediastinal lymph nodes including the thymus whereas the infradiaphragmatic modified "inverted y" field encompasses the spleen and para-aortic, inguinal and iliacal lymph nodes while shielding non-lymphoid tissues in the head, chest (lung, breast), abdomen (liver, kidneys) and pelvis (bladder, rectum). Seven Gray (Gy) single dose TNI was combined with anti-T lymphocyte antibody OKT3 (n=11), antithymocyte globulin (ATG) (n=12), fludarabine (n=13), thiotepa (n=11), cyclophosphamide (n=2), and etoposide (n=1) followed by an infusion of peripheral blood stem cells. Twelve patients had haploidentical family donors, one child had a HLA-C mismatched unrelated donor and one child had a sibling donor. All children received T-cell depleted grafts. Various GvHD prophylaxis were employed and comprised ciclosporine A, mycophenolatmofetil, tacrolismus, basiliximab, prednisolone and methylprednisolone. *Results.* The median interval between initial transplantation and retransplantation was 41 days (range, 31-93 days). All patients were transplanted in aplasia. Engraftment was seen in all children after a median of 10 (range 9-32) days with sustained complete donor chimaerism in 13/14 children. One child receives donor lymphocyte infusions due to mixed chimaerism. TNI-based reconditioning was well-tolerated with no severe toxicity. All patients had mild to moderate mucositis and fever. In contrast, no permanent constraint of lung function and clinical signs of veno-occlusive disease were detected. In addition, no cardiac or neurotoxicity occurred. After a median follow-up of 928 (range 27-2550 days), disease-free and overall survival rates are 64%/71%, respectively. Despite sustained engraftment, 4 patients died due to disease relapse (n=3) and Moschkowitz syndrome (n=1) and one child is alive with MDS relapse. *Summary/Conclusions.* In patients with graft failure or graft rejection after allogeneic HSCT, TNI-based reconditioning regimens allow sustained engraftment paralleled by favourable toxicity profile potentially leading to long-term overall/ disease-free survival. Our results are encouraging since graft failure or early graft rejection have been fatal for the majority of patients.

1093

UNRELATED DONOR IS COMPARABLE WITH SIBLING DONOR AS A DONOR TYPE FOR PERIPHERAL BLOOD STEM CELL TRANSPLANTATION IN ACUTE MYELOID LEUKEMIA PATIENTS

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Background. The immune cell profiles of G-CSF mobilized peripheral blood cells are different from those of bone marrow cells. Therefore, the post-transplant immune mediated complications and transplant outcomes can differ between bone marrow transplantation and peripheral blood stem cell transplantation. Although the outcomes of sibling donor bone marrow transplantation are generally better than those of unrelated donor bone marrow transplantation, there has been no comparison study in a single disease between sibling and unrelated donor peripheral blood stem cell transplantation (PBSCT) setting. *Aims and methods.* We performed a retrospective study for adult acute myeloid leukemia patients who had received an allogeneic PBSCT from an HLA-matched sibling or unrelated donor to compare the transplant outcomes and post-transplant complications between sibling and unrelated donor PBSCT. The median follow up duration was 23.7 months. *Results.* The neutrophil and platelet recovery were not different between the two groups. The cumulative incidence of acute GVHD at day 100 posttransplant was higher in unrelated donor PBSCT (59.8% *vs.* 32.3% in sibling donor PBSCT; P=0.003), while the cumulative incidence of chronic GVHD at two years was not different between sibling and unrelated donor PBSCT (83.7% *vs.* 61.3%; P=0.113). There were no statistically significant differences in disease-free survival (sibling donor: 51.9% *vs.* unrelated donor: 55.4%; P=0.961), overall survival (sibling donor: 55.9% *vs.* unrelated donor: 58.8%; P=0.524), transplant-related mortality (sibling donor: 30.9% *vs.* unrelated donor: 26.7%; P=0.554), and relapse incidence (sibling donor: 55.9% *vs.* unrelated donor: 58.8%; P=0.524) at two years. Multivariate analysis indicated that chronic GVHD was favorable prognostic factor (HR = 0.206; 95% CI = 0.117-0.576; P=0.001) and pre-transplant disease state (>CR1) was unfavorable prognostic factor (HR=2.279; 95% CI=1.070-4.851; P=0.033) for DFS. *Conclusions.* Although unrelated donor PBSCT was associated with a higher risk of acute GVHD, the risk of chronic GVHD was not different between the two groups. The transplant outcomes were not different between sibling and unrelated donor PBSCT in AML patients. The unrelated donor was comparable with sibling donor as a donor type for PBSCT.

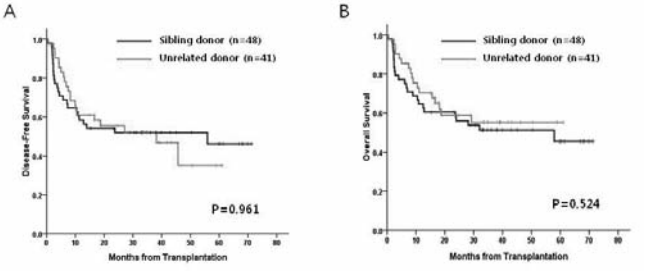

Figure. Disease-free survival (A) and overall survival (B) in allogeneic peripheral blood stem cell transplantation from sibling and unrelated donor.

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INTRAVENOUS BUSULPHAN IN COMBINATION WITH ETOPOSIDE AND MELPHALAN USED AS CONDITIONING REGIMEN FOR AUTOLOGOUS HEMATOPOIETIC CELL TRANSPLANTATION IN LYMPHOMAS: EVALUATION OF TOXICITY

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Background. The standard conditioning regimen used in autologous hematopoietic cell transplantation (autoHCT) for lymphomas is BEAM. During the last twelve months, due to carmustine (BCNU) unavailability, the combination of busilvex (3.2 mg/kg/day for 3 days), etoposide (400 mg/m^2/day for 2 days) and melphalan (140 mg/m^2) was used as conditioning regimen. *Aim.* Evaluation of the safety of the above regimen.

Methods. We retrospectively evaluated 20 patients, aged 27 (16- 65) years, transplanted for Hodgkin (12) and non Hodgkin Lymphomas (DLBCL 5; large B cell mediastinal 1; T-anablastic 1; MCL 1) between May 2009 and February 2010. The patients received a median of 3 (2-5) treatment lines before autoHCT. Disease status was refractory in 12, relapsed in 5, and complete remission (CR) in 3 (1 CR1, MCL; 2 CR2). According to the Hematopoietic cell transplantation specific comorbidity index, 15 were low (score 0) and 5 intermediate (score 1) risk. In 19 mobilization was successful after chemotherapy and GCSF (dexamethasone, ifosfamide, cisplatin, etoposide - DICE 16, DHAP 1, mini BEAM 1, R-DICE 1) and in 1 with GCSF plus AMD3100 (plerixafor) due to poor mobilization with DICE/GCSF. Fifteen/20 received palifermin and all received cotrimoxazole, antifungal, antiviral and antibiotics as prophylaxis for at least 3 months. The median $CD34^+$ cell dose was 5.65 (1.7-19.59) $\times 10^6$/Kg and patients received GCSF for a median of 9.5 (7- 53) days. *Results.* The median time for neutrophil engraftment (ANC> 500) was day +11 (9- 31), +13 (9 days- 5 months) for platelet (>20000) and +11 (8- 43) for erythroid engraftment (transfusion independency) in 19/20 patients. A second transplantation with double cord blood was offered in one patient because of engraftment failure. In one patient stable engraftment was achieved after a second infusion of autologous cells due to delayed engraftment. The median number of red blood cell transfusions were 3 (0-15); median single donor platelet units were 3.5 (0- 36). Infection during conditioning developed in 3 (urinary 2, respiratory 1) and during neutropenia developed in 15 patients (bacteremias mostly; no fungal or respiratory infections). Patients were febrile for a median of 4 (1-42) days. Nineteen developed mucositis (grade I 5, II 7, III 7) and 16 received parenteral nutrition for 5.5 (5-12) days. Eighteen developed liver and gastrointestinal toxicity (elevation of transaminases, nausea/vomiting) grade I- III. The median day of discharge was +14 (10- 32) in 19/20 patients. The patient allografted post autoHCT died at day +63 due to refractory disease and infections. The median follow-up was 4 (1-11) months. Eighteen/20 patients are alive. Eight/18 (44%) were evaluated at +3 months with PET scan and are in CR. Two/18 are in refractory/progressive disease, 1/18 in CR, 6/18 in stable disease without further treatment and 1/18 is not evaluated yet. Two/20 patients died; one due to refractory disease and another due to refractory disease and infection post allogeneic transplantation. *Summary/Conclusions.* The toxicity of the conditioning regimen seems acceptable, but no definite conclusions can be made on efficacy, because of the short follow up and limited number of patients.

SIMULTANEOUS SESSION II

Myeloma and other monoclonal gammopathies - Clinical 2

1095

THALIDOMIDE MAINTENANCE SIGNIFICANTLY IMPROVES PROGRESSION FREE (PFS) BUT NOT OVERALL SURVIVAL (OS) OF MYELOMA PATIENTS, WITH PFS BENEFITS IN FAVOURABLE FISH SUBGROUPS ONLY: MRC MYELOMA IX RESULTS

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Background. The role of maintenance thalidomide in myeloma remains unclear, with other major studies reporting some effect on PFS, but with variable results on survival. Also, the impact of maintenance dependent on patient risk group has not been fully evaluated, with the Arkansas group having shown a survival benefit in poor prognosis patients with cytogenetic abnormalities, while in contrast the IFM group showed a benefit in a study restricted to good prognosis patients defined by B2M and chromosome 13 deletion. *Aims.* The study aimed to randomise 762 patients with myeloma to maintenance thalidomide or not, with 80% power to detect a 10% difference in five-year OS, and with PFS as a joint primary endpoint. *Methods.* Following induction treatment in an intensive pathway for younger/fitter patients, and a non-intensive pathway for the remaining patients, the MRC Myeloma IX Study randomised 820 patients to either maintenance thalidomide, aiming for an initial dose of 50 mg increasing to 100mg daily, or to no maintenance, with 818 patients evaluable. Patients of all ages were randomised (median age 65, range 31-89). FISH was performed using standard approaches for t(4;14), t(6:14), t(11;14), t(14;16), t(14;20), del (13), gain 1q, del1p32 and del 17p. Written informed consent was obtained at the outset for all patients. ISRCTN6845411.

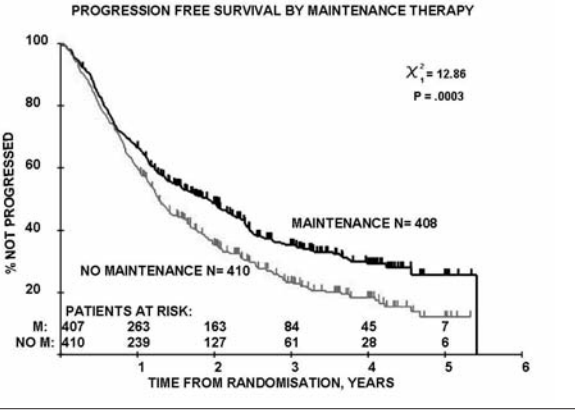

Figure.

Results. Median follow-up from maintenance randomisation was 3.2 years, range 1 year to $5^1/_2$ years. The results show that maintenance thalidomide significantly improves PFS (Figure), with a difference between the curves of 13% (95% CI 6%-20%) established by two years and maintained to the current maximum follow up at $5^1/_2$ years, logrank P=.0003, HR 1.36 (95% CI 1.15-1.61). However, there was no impact on OS (logrank P=.40). For the 61% of patients who had FISH evaluated, there were differing effects dependent on the FISH group. Defining adverse FISH groups as any of t(4;14), t(14;16), t(14;20), 1p32-, 1q+ or 17p-, and the rest as favourable, the PFS benefit was seen in the favourable FISH group (P=.05) with no effect on OS (P=.94). In the adverse FISH group there was no effect on PFS (logrank P=.98) and OS appeared to be impaired, though the result was not significant (n=127, logrank P=.07). To understand the impact of these potentially important beneficial effects on PFS we looked at survival after relapse. In this analysis there was a significant negative impact of maintenance thalidomide on survival after relapse, logrank P=.005, more pronounced in the

adverse FISH group (logrank P=.004), although there was still some initial separation of the curves in the favourable FISH group (logrank P=.35 but a 15% difference at 18 months). This could, at least partly, be attributable to the lack of an effect of thalidomide at relapse for those patients previously treated with maintenance thalidomide and the lack of availability of bortezomib or lenalidomide as alternative treatments. *Conclusions.* Maintenance thalidomide significantly improves PFS, particularly in favourable prognosis myeloma defined by FISH analysis, although there is no impact on OS due to poorer survival after relapse, particularly in adverse prognosis myeloma defined by FISH analysis.

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IMPACT OF UPFRONT BORTEZOMIB-BASED REGIMENS ON CLINICAL OUTCOMES OF NEWLY DIAGNOSED MULTIPLE MYELOMA PATIENTS ACCORDING TO CYTOGENETIC ABNORMALITIES BY FISH ANALYSIS

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The role of novel agents for the treatment of newly diagnosed multiple myeloma (MM) patients with high-risk cytogenetic abnormalities [t(4;14) and/or del(17p)] is still controversial. To more carefully address this issue, we analyzed the clinical outcomes of a large series of MM patients who received bortezomib as part of upfront induction therapies. Regimens evaluated were bortezomib-thalidomide-dexamethasone (VTD), bortezomib-melphalan-prednisone (VMP) and bortezomib-melphalan-prednisone-thalidomide (VMPT). A total of 587 patients who could be evaluated for del(13q), t(4;14) and del(17p) by interphase FISH were included in the present analysis. Study end points included achievement of immunofixation negative complete response (CR) and progression-free survival (PFS). Three cytogenetic subgroups of patients were identified, including those without any of the three genomic abnormalities (n=261), with del(13q) alone (n=174) and those with t(4;14) and/or del(17p) (n=152). Overall, the frequency of patients with no abnormalities, with del(13q) alone and with t(4;14)±del(17p) was 45.8%, 29.8% and 24.3%, respectively. No statistically significant difference in terms of CR rate was detected by comparing high-risk patients with those who lacked cytogenetic abnormalities (38.1% *vs.* 32.5%; P=0.2) or who carried del(13q) alone (46.5%; P=0.1). By the opposite, the CR rate in patients with del(13q) alone was significantly higher than among those without cytogenetic abnormalities (P=0.003). The 30-month projected PFS was 62% (27.6% events) for patients with high-risk cytogenetics *vs.* 66% (19.5% events) for those without abnormalities (P=0.06) *vs.* 64% (21.2% events) for those with del(13q) alone (P=0.5). To more carefully investigate the impact on prognosis of different high-risk cytogenetic abnormalities, we compared the clinical outcome of patients with t(4;14) but lacking del(17p) with that of patients who were del(17p) positive but t(4;14) negative. These latter patients had a significantly lower probability to achieve CR (28.3%) than those who were t(4;14) positive but del(17p) negative (47.6%) (P=0.02). By the opposite, the 30-month PFS for these two subgroups of patients was almost superimposable, with values at 66% (26.4% events) and 69% (21.4% events), respectively (P=0.3). These results, based on a post-hoc analysis, should be cautiously interpreted, although consistencies exist between data herein shown and previous reports on the activity of bortezomib in patients with high-risk cytogenetic abnormalities. Further analyses of homogeneously treated series of patients are warranted to draw firm conclusions about the ability of bortezomib-based regimens to overcome the adverse prognosis related to the presence of t(4;14) and/or del(17p).

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BORTEZOMIB PLUS DEXAMETHASONE VERSUS REDUCED-DOSE BORTEZOMIB PLUS THALIDOMIDE-DEXAMETHASONE AS INDUCTION PRIOR TO AUTOLOGOUS TRANSPLANTATION IN NEWLY DIAGNOSED MYELOMA

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Background The Intergroupe Francophone du Myelome (IFM) has shown that induction treatment prior to ASCT with VD is superior to VAD in terms of complete response (CR) or CR plus very good partial response(VGPR) both before and after ASCT (Harousseau 2008 Joint ASH/ASCO session). The Italian and the Spanish groups have presented impressive results with the three-drug combination velcade,thalidomide,dexamethasone (VTD)(Cavo Blood 2009;114:148 and Rosinol 2009,114:59) However in these studies, bortezomib-associated peripheral neuropathy (PN)was frequent and sometimes severe (9-14% Grade 3). *Aims.* To show that with reduced doses of bortezomib, vTD is superior to TD and better tolerated. *Patients and Methods.* The IFM has conducted a randomized trial comparing four 21-day cycles of induction with VD(V1.3 mg/m^2/d on days 1,4,8,11 plus D 40mg:d on days 1-4 and 8-11 for the first 2 cycles, on days 1-4 for the last 2 cycles) or vTD (v 1mg/m^2 /d on days 1,4,8,11 plus thalidomide 100mg:d d1-21 plus dexamethasone same dosing as for VD). Results were assessed after cycle 2 and 4 and after ASCT. Responses were evaluated according to IMW uniform criteria . Samples for serum and urine electrophoreses were centralized (HAL,CHU Nantes). In the vTD arm, if after cycle 2, the response was<PR, the doses of v and T were to be increased to 1.3 mg/m^2 and 200mg/d respectively (in the absence of PN). *Results.* From 03/2008 to 01/2009, 205 patients with newly diagnosed symptomatic MM and up to 65 years of age were recruited and randomized at diagnosis (stratification according to β-2 microglobulin and presence of del(13) by FISH). As of December 2009, 191 patients are evaluable for response after cycle 4 (95 VD,96 vTD). According to investigators'assessment,the efficacy results are the following (VD *vs.* vTD): CR 12%vs 14% (P=0.68),≥VGPR 36%vs 50 % (P=0.047), ≥PR 81%vs 90%(P=0.09),stable disease 12%vs 5%, progression/failure 7%vs 4%.In the vTD arm the doses of v and T were increased due to <PR after cycle 2 in only 7 patients. The post-ASCT results are the following CR 26% *vs.* 20% (P=0.33),[3] VGPR 54%*vs.* 66% (P=0.044)[3] PR 84% *vs.* 92% (P=0.33). Bortezomib treatment was reduced or interrupted due to toxicity in 17 % of cases in the VD arm and 7% of cases in the vTD arm. There was no difference in the incidence of adverse events and of severe adverse events. There were 4 severe adverse events related to PN in the VD arm *vs.* 0 in the vTD arm . The proportion of patients with at least one report of PN was: all grades 63% *vs.* 56%(P=0.39);Grade ≥ 2 28 % *vs.* 16 % (P=0.04), Grade ≥36% *vs.* 3 % (P=0.3). *Conclusion.* The combination of reduced-dose bortezomid and thalidomide induces significantly more CR+VGPR than VD with usual doses of V. Despite the addition of thalidomide, the incidence of PN was markedly reduced In the vTD arm with only 3% Grade ≥3 PN. vTD should be considered a new standard for induction treatment prior to ASCT.

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FINAL DONOR VERSUS NO DONOR COMPARISON OF NEWLY DIAGNOSED MYELOMA PATIENTES INCLUDED IN THE HOVON 50/54 STUDY

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Background. The role of Allogeneic Stem Cell Transplantation (Allo-SCT) in myeloma is disputed. Its value should be determined by prospective randomized trials. It is widely accepted that for the evalu-

ation of treatment results of Allo-SCT genetic randomization based on the availability of an HLA identical sibling donor is a reliable surrogate for such a truly randomization. *Aims.* To evaluate the value of nonmyeloablative Allo-SCT as part of first line therapy. *Methods.* Patients with an HLA-identical sibling donor, included in the phase-III HOVON50 study, that was designed to assess the effect of thalidomide in induction treatment and as maintenance after high-dose therapy (HDM 200 mg/m^2) and auto-SCT could proceed to the Hovon 54 study in which an Allo-SCT after conditioning with low dose TBI only was performed between 2-6 months after HDM. 122 patients could be classified as having a sibling donor of which 100 patients underwent the Allo transplant and139 patients had no sibling donor of which 122 patients started with maintenance therapy. Both groups were comparable with regard to age, myeloma stage, and prognostic factors including cytogenetics and ISS stage. *Results.* 95% of the patients in the no donor group achieved at least a PR, including 75% of patients with a VGPR and 20% with a CR, *vs.* 96% of the patients in the donor group including 35% CR and 70 % VGPR. After a median follow-up of 60 months after HDM, PFS and OS were comparable between the two groups. Median PFS was 30 *vs.* 28 months for the donor group and no donor group respectively (HR 0.8, CI:0.6-1.1). Median OS was not reached with at 60 months 58% of patients alive in the donor group *vs.* 60% of patients in the no donor group (HR 1,27, CI 0.85-1.89). For patients that did receive their allocated transplant or maintenance treatment median PFS was 29 months (HR 0.76,CI: 0.54-1.06) and OS at 60 months from HDM was 60% for the donor group *vs.* 55 % for the no donor group (HR 0.92, CI 0.58-1.45). *Conclusion.* This analysis showed no improvement of tandem Auto Allo-RIC as part of first line therapy in myeloma as compared Auto-SCT followed by maintenance therapy.

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RESULTS OF PX-171-004, AN ONGOING OPEN-LABEL, PHASE II STUDY OF CARFILZOMIB IN PATIENTS WITH RELAPSED AND/OR REFRACTORY MULTIPLE MYELOMA (R/R MM) WITH OR WITHOUT PRIOR BORTEZOMIB EXPOSURE

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Background. Carfilzomib (CFZ) is a proteasome inhibitor with unique target selectivity and an irreversible binding mechanism that results in sustained proteasome suppression. In preclinical studies CFZ overcomes bortezomib (BTZ)-resistance *in vitro* (Kuhn *et al.*, Blood 2007) and, lacks non-proteasome off-target activities associated with BTZ (Kapur *et al.*, Blood 2008). This may account for observed differences in tolerability of CFZ (e.g. low neuropathy rates), permitting consecutive day dosing and treatment over extended periods of time. In a previous Phase II study (PX-171-003), single-agent CFZ achieved durable responses and maintained stable disease in patients with relapsed/refractory multiple myeloma (MM) despite prior resistance to all available agents, including BTZ. *Aims.* We present here updated data from PX-171-004, an ongoing Phase II study of single-agent CFZ in MM patients with relapsed or refractory disease (R/R MM) following 1-3 prior therapies with emphasis on the BTZ-naïve and BTZ-treated cohorts. *Methods.* Patients with relapsed or refractory (defined as <25% response or disease progression during therapy) MM were enrolled and stratified based on prior BTZ exposure (i.e., BTZ-naïve and BTZ-treated) and CFZ dose level (see Table). For the BTZ-treated cohort, tolerability and prior response to BTZ were not an eligibility requirement. CFZ 20 or 27 mg/m^2 was administered on days 1, 2, 8, 9, 15 and 16 every 28 days, for up to 12 cycles. The primary endpoint was overall response rate (ORR; ≥ partial response [PR]) by IMWG criteria. Secondary endpoints included clinical benefit response (CBR; ≥minimal response [MR]), duration of response (DOR), and safety. *Results.* For BTZ-naïve patients, cohort 1 (20 mg/m^2) included 59 patients and cohort 2 (27 mg/m^2) included 20 patients. Patients received a mean of 5 cycles of CFZ (range 1-12). The BTZ-treated cohort included 35 patients (14 refractory to most recent treatment). For BTZ-naïve patients, 54 patients in cohort 1 and 19 patients in cohort 2 were evaluable with ORRs of 46% and 53%, respectively. Median DOR (≥MR) was ≥8.8 months (mo). Thirty-three patients previously treated with BTZ were also evaluable with an ORR of 18% (Table). In the combined patient population, the most common adverse events (AEs) included fatigue (66%), nausea (49%), dyspnea (40%), anemia (34%), diarrhea (32%) and were primarily ≤ Grade 2. The most common ≥ Grade 3 AEs were anemia, thrombocytopenia, and neutropenia (10% each). Febrile neutropenia (all Grades, 2%; ≥ Grade 3.2%) and treatment-emergent peripheral neuropathy (PN) (all Grades, 6%; ≥ Grade 3, 1%) were uncommon. Of 23 patients (21%) who completed all 12 cycles of the protocol, 7 chose to continue CFZ therapy on an extended treatment protocol. *Conclusions.* Single-agent CFZ is active in patients with R/R MM with 53% of BTZ naïve patients responding to 20 mg/m^2 in cycle 1 followed by 27 mg/m^2 thereafter. AEs were generally mild and durable disease control was achieved with continued dosing. Severe PN is rare (≥ Grade 3, 1%) and does not limit therapy, despite pre-existing PN. These data support the continued evaluation of CFZ as a safe and effective treatment option in MM.

Table. Treatment groups and patient responses.

	BTZ-treated	BTZ-naïve	
	N=35	Cohort 1 N=54	Cohort 2 N=19
CFZ Administration	20 mg/m^2	20 mg/m^2	20 mg/m^2 cycle 1, 27 mg/m^2 cycle 2+
Best Response	n (%)	n (%)	n (%)
CR	1 (3)	1 (2)	0 (0)
VGPR	1 (3)	5 (9)	1 (5)
PR	4 (11)	19 (35)	9 (47)
MR	4 (11)	8 (15)	0 (0)
SD	13 (37)	12 (22)	6 (32)
Median DOR (≥MR)	9.0 mo	8.8 mo	TBD
Median TTP	5.3 mo	7.6 mo	TBD

Myeloproliferative disorder - Biology

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TRANSGENIC MICE EXPRESSING A JAK2 EXON 12 MUTATION DISPLAY ISOLATED ERYTHROCYTOSIS CLOSELY RESEMBLING THE HUMAN JAK2 EXON 12 POLYCYTHEMIA

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Background. JAK2-V617F mutation and JAK2 exon 12 mutations together account for more than 98% of patients with polycythemia vera (PV). Mutations in exon 12 of JAK2 are detected in approximately 50% of PV patients that are negative for JAK2-V617F. *Aims.* To study the *in vivo* role of JAK2 exon 12 mutations in the pathogenesis of PV, we generated inducible transgenic mouse strains carrying the most frequent exon 12 mutation (JAK2-N542-E543del). *Methods.* A human JAK2 gene with the sequences of exon 12 were placed in the inverse orientation and flanked by antiparallel loxP sites. Recombination of loxP sites by Cre recombinase results in flipping of exon 12 sequence, restoring a functionally active transgene. *Results.* Three transgenic mouse lines (E12-N1, E12-N2 and E12-N3) were established. Real-time PCR analysis revealed that E12-N1 had 2 copies, E12-N2 had 5 copies and E12-N3 had 7 copies of the transgene. These strains were crossed with MxCre transgenic mice that allow induction of Cre expression and thus activation of the JAK2 transgene by injection of polyinosine-polycytosine (pIpC). All three mouse lines developed PV-like phenotype within 12 to 16 weeks after pIpC injection, with significant increase in red blood cells, hemoglobin, and hematocrit. Interestingly, unlike the PV-like phenotype induced by JAK2-V617F, which in addition to increase in red blood cell parameters also showed thrombocytosis and neutrophilia, the PV-like phenotype induced by JAK2-N542-E543del had neutrophils within normal range and platelet counts that were normal or even slightly decreased. This phenotype closely resembles clinical features of human PV patients with JAK2 exon 12 mutations, who usually have normal counts of white blood cells and platelets. The PV phenotype in JAK2-V617F transgenic mice was only present with higher expression levels of JAK2-V617F. However, the expression levels of the N542-E543del transgene were markedly lower than those of the JAK2-V617F, suggesting that qualitative differences in the signals generated by the two mutated Jak2 proteins may exist. *Summary.* Since JAK2-V617F and JAK2-N542-E543del both signal through the same tyrosine kinase domain, these results raise the interesting question why the phenotypes are different. Since the genetic background of these mice is identical, the JAK2-V617F and N542-E543del transgenic mice now allow examining the molecular basis for the phenotypic differences between these two PV subtypes.

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PEGYLATED INTERFERON-ALFA (IFNA) 2A TREATMENT TARGETS JAK2V617F CLONES WITHOUT AFFECTING TET2 MUTANT CELLS IN POLYCYTHEMIA VERA (PV)

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Background. In a phase 2 trial of peg-IFNa-2a, we observed a substantial decrease of *JAK2V617F* allele burden in granulocytes from 29 patients treated with IFNa, including complete molecular remissions (CMR) in 7/29. These 7 CMR constitute the first homogeneous cohort of patients with measurable reduction of the PV clone, which may allow answering whether such CMR are associated with elimination of the PV clone. *Methods.* We performed bone marrow (BM) erythroid progenitor cultures (using BMMC in plasma clot, and purified CD34$^+$ cells in methylcellulose assay) and *JAK2* and *TET2* genotyping of individual colonies from 5 patients in CMR after 18 to 40 months of pegIFNa-2a treatment. *Results.* At *JAK2V617F* CMR, few residual endogenous erythroid colonies (EEC) were still found in the 5 patients, representing only 0.2 to 6% of the total of colonies grown with Epo. In 1 patient with 3 sequential BM samples, numbers of EEC continuously decreased during IFNa, and no residual *JAK2V617F* positive colonies were detected after 34 months. In 1 patient, we found a single nucleotide deletion in exon 11 of *TET2* leading to a frameshift (FS) and a premature stop codon. At baseline, both mutant *TET2* and *JAK2* represented 20% of their wild-type (WT) counterparts. The *TET2* mutated and *JAK2V617F* clones didn't decrease in parallel, the burden of mutant *TET2* remaining unchanged in 2 samples taken after *JAK2V617F* CMR (after 24 and 36 months of IFNa). We next sequenced *TET2* in *JAK2V617F* positive and negative colonies grown from CD34$^+$ cells at time of peripheral *JAK2V617F* CMR. *TET2* mutation was found in 1/5 (20%), and 10/12 (83%) of *JAK2V617F* positive, and negative colonies, respectively. Presence of JAK2V617F homozygous/ *TET2WT* and *TET2FS* homozygous/ *JAK2WT* colonies, suggested occurrence of mitotic recombinations or deletions on *JAK2* and *TET2* loci in separate clones. Genotyping of informative SNPs in each gene suggested that two separate primary clones coexisted. The first clone initially acquired *JAK2V617F* giving rise to *JAK2V617F / TET2WT* colonies, which next generated a subclone with homozygous *JAK2V617F* mutation. The second clone acquired the *TET2FS* mutation and generated two subclones: one that lost *TET2WT* allele, and one that secondarily acquired *JAK2V617F*. We found a striking discrepancy in the frequency of *TET2 vs. JAK2* mutant cells after therapy whereas both mutations had the same allelic burden before IFNa: 11/17 (65%) colonies carried the *TET2* mutation in contrast to 7/89 (5%) with *JAK2V617F* suggesting that IFNa targets *JAK2V617F* cells without affecting *TET2* mutant cells. *Conclusion.* We showed that IFNa therapy led in all studied patients to a dramatic decrease in the size of the clone harboring the JAK2 mutation, including complete elimination of *JAK2* mutant colonies in 1/5 patient. In contrast in another patient with a biclonal *TET2/JAK2* disease, IFNa therapy had a drastic effect on *JAK2* mutated clones but did not affect the TET2 mutant clone. As this patient achieved hematologic and *JAK2* molecular complete remissions on IFNa without reduction of the *TET2* clone we hypothesize that this treatment brought the patient back to a 'pre-proliferative' phase of his disease.

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V617F MUTATION INDUCES NUCLEAR LOCALIZATION OF JAK2 IN CD34+ CELLS BUT NOT GRANULOCYTIC, MEGAKARYOCYTIC OR ERYTHROID CELLS OF PATIENTS WITH PHILADELPHIA-NEGATIVE MYELOPROLIFERATIVE DISORDERS

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Background. Recently, Dawson *et al.*. identified a previously unrecognized nuclear role for JAK2 in the phosphorylation of the tyrosine 41 of the histone H3 with the exclusion of HP1a from chromatin and resulting in a disregulation of several JAK2-regulated genes such as IMO2 in haematopoietic cell lines and in one case on peripheral CD34+ cells from a JAK2V617F mutated primary myelofibrosis (PMF) patient. Activation of JAK2 by chromosomal translocations or point mutations is a frequent event in haematological malignancies particularly in Philadelphia negative myeloproliferative disorders (MPDs). *Aims and Methods.* To investigate and confirm a possible nuclear localization of JAK2 in presence of V617F mutation, we stably transfected K562 with pMSCV-Puro-JAK2V617F construct and compare with K562 expressing pMSCV-Puro-wild type-(WT)-JAK2 and performed immunofluorescence and western blot analysis. To confirm the *in vitro* results we searched the possible nuclear localization of JAK2 in total BM of 10 patients affected by all types of JAK2V617F positive MPDs [PMF n= 3, polycitemia vera (PV) n= 3, essential thrombocythemia (ET) n= 4] and 5 patients with WT MPDs (PMF n= 2 , ET n=3). To define which cells show nuclear JAK2, we selected by fluorescence activated cell sorting (FACS) 4 cell populations: CD34$^+$, CD15$^+$, CD41$^+$ and CD71$^+$ cells from total BM of 3 JAK2-mutated-MPDs (1 ET, 1 PV, 1 early PMF). *Results.* Confocal immunofluorescent images on nuclear and cytoplasmic fractions confirmed nuclear JAK2 in K562 although with the strongest nuclear signal in JAK2V617F expressing cells. This latter was also seen by western blot analysis which showed nuclear and cytoplasmic JAK2 only in JAK2V617F expressing K562 comparing with untransfected and WT cells. No differences in JAK2 nuclear signal was observed by the addiction of the nuclear export inhibitor leptomycin B suggesting that export is not involved in nuclear JAK2 shuttling. We found a strong nuclear signal within the nuclei of 3-5% of mononucleated cells in 10 of 10 JAK2 mutated patients but not in un-mutated cases. We found nuclear JAK2 in CD34$^+$ cells but not in other cell populations of the 3 studied patients. Western blot performed on nuclear and cytoplasmic fractions of the JAK2V617F-CD34$^+$ cells confirmed the result. No nuclear JAK2 was detected in differentiated erythroid, granulocytic or megakaryocytic

colonies obtained from all the studied patients (n=15). *Conclusions.* Our data corroborate the recent findings, obtained in hematopoietic cell lines, of a role of JAK2 in direct nuclear signaling. Furthermore we report, for the first time, a nuclear JAK2 in total BM and in sorted CD34+ cells of patients affected by all subtypes of JAK2 mutated MPDs and not in patients with WT diseases. We described also the absence of nuclear JAK2 in sorted mature cells and in differentiated colonies derived from the same patients. Possible chromatin modification due to JAK2 nuclear localization has to be better assessed in patients, where further studies are needed to understand the effects of mutated JAK2 in the nuclei, its target proteins and consequences on gene expression. This intriguing insight reveals a new scenario in the pathogenesis of malignant haematopoiesis and in myeloproliferative phenotype.

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SIGNALING PATTERNS IN MYELOPROLIFERATIVE NEOPLASMS SEGREGATE WITH DISEASE PHENOTYPE RATHER THAN JAK2 GENOTYPE

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The recent discovery of acquired mutations in signaling pathways, such as the JAK2 V617F mutation, has provided a molecular basis for the pathogenesis of the myeloproliferative neoplasms (MPN). However, the nature of aberrant signaling in primary cells from MPN patients remains poorly understood. Most work has been described in cell lines or in animal model systems which may not adequately reflect the cellular context or the molecular complexity of human MPN. In addition where patient material has been used, technical aspects limit the interpretation of these experiments. We undertook a detailed and physiological characterization of the signaling abnormalities in human MPN to understand how these abnormalities contribute to the cellular characteristics and disease phenotype. We first developed a reproducible and quantitative "Phosphoflow" assay to measure levels of the activated signaling intermediates phospho-STAT5, phospho-STAT3, phospho-AKT and phospho-ERK 1/2, using intracellular staining with phospho-specific-antibodies and detection with flow-cytometry. This allowed us to consistently interrogate signaling in both the CD34+ hematopoietic stem and progenitor compartment (HSPC) and the CD34- maturing cell compartments in up to 27 MPN patients (10 PV, 9 ET and 8 MF) comparing them to similar compartments from the bone marrow of 6 normal controls. In addition, we also studied dynamic signaling in MPN patient samples following stimulation with EPO or inhibition with a JAK2 inhibitor and correlated signaling with disease characteristics such as clonal burden and stem cell, progenitor and later compartment size. Our results demonstrated significant activation of the STAT5, ERK and STAT3 pathways in MPN patients over normal controls. Aberrant signaling was detected in both the CD34+ and CD34- compartments, but often varied between these compartments in individual diseases. In contrast, compartment size only increased in later compartments. The pattern of aberrant signaling correlated with the disease phenotype (PV, ET or MF) but not the JAK2 mutations status of MPN patients. In addition, although EPO stimulation increased STAT5 signaling in normal controls MPN samples were maximally stimulated. MPN samples also showed modest inhibition of signaling with TG101209, a JAK2 specific inhibitor in both the CD34+ and CD34- compartments and the degree of inhibition was similar to that seen in CML patient cells treated with Imatinib. However, this inhibition was not JAK2 V617F specific, as MPN patients with wild-type JAK2 and normal control patients were inhibited to a similar degree. Finally, JAK2 V617F mutant burden demonstrated a poor correlation with signaling and compartment size. Our results provide the first systematic demonstration of specific intracellular signaling events in human MPN samples. Our data also demonstrate that JAK2 status and mutation burden correlate poorly with disease and cellular phenotype. We further demonstrate only modest inhibition of cellular signaling with a JAK2 inhibitor which occurs in both MPN and normal samples. This suggests that other factors, such as germline modifiers or other co-existing mutations, significantly contribute to the MPN phenotype. Taken together our study has major implications for both the pathogenesis and therapy of MPN.

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OVEREXPRESSION OF TRANSCIPTION FACTOR NF-E2 IN VIVO CAUSES A MYELOPROLIFERATIVE DISORDER WITH EXPANSION OF THE STEM CELL COMPARTMENT

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Background. The transcription factor nuclear factor erythroid-2 (NF-E2) is expressed in hematopoietic stem cells as well as in myeloid, erythroid and megakaryocytic precursors. It is known to play an important role in both erythropoiesis and platelet formation as NF-E2 deficient mice display marked anemia at birth and die perinatally due to thrombopenia, However, its role in hematopoietic stem cells has not been studied in detail. We have previously shown that NF-E2 is overexpressed in the vast majority of patients with Myeloproliferative Neoplasms (MPNs), a group of acquired disorders of the hematopoietic stem cell (HSC). Recently we have engineered a transgenic mouse model, in which NF-E2 is overexpressed specifically in the hematopoietic compartment including the HSCs. *Aims.* We therefore investigated the effect of NF-E2 overexpression on the HSC pool. *Methods.* Two independent founder strains were generated, which express NF-E2 under control of the vav promoter. Hematological parameters were determined by CBC every two months. Colony formation in the bone marrow was assessed by growth in methylcellulose media (Stem Cell Technologies). Subfractions of HSC were displayed by FACS analysis using the following 7-color stain: Lin-cocktail-FITC, c-kit-APC-eFlour780, Sca-1-PacificBlue, CD34-AlexaFlour647, Fc-gamma-II/III-R-PE-Cy7, Thy1.1-PerCP, Flt3-PE. *Results.* NF-E2 transgenic (tg) mice develop thrombocytosis with a latency of 12-14 months. Concomitantly, megakaryocyte colony formation in the bone marrow is drastically increased. In addition, Epo-independent colony formation, a pathognomonic feature of polycythemia vera, is significantly increased in NF-E2 transgenic animals. Bone marrow histopathology shows findings characteristic of MPNs, including increased numbers of megakaryocytes with cytologically abnormal forms, often in clusters. Paralleling the histological observation, total bone marrow cellularity was significantly increased in NF-E2 transgenic mice (wt: $5.1 \pm 1.6 \times 10^6$ cells/femur *vs.* tg: $7.2 \pm 1.8 \times 10^6$ cells/femur; $P<0.05$). The stem cell compartment was further characterized using FACS analysis of phenotypically defined subfractions. Both the KSL ($kit^+/sca\text{-}1^+/lin^-$) and the KL ($kit^+/sca\text{-}1^-/lin^-$) compartments were significantly increased in NF-E2 tg mice (KSL as % of lin--cells: wt: 5.3 ± 2.7%, tg: 9.0 ± 5.4%, P=0.005; KL as % of lin--cells: wt: 19.2±3.9%, tg: 23.6 ± 6.2%; P=0.02). In addition, both the percentage and the absolute numbers of LT-HSCs (long-term HSCs) was significantly elevated in NF-E2 tg mice (LT-HSC as % of lin--cells: wt: 0.33 ± 0.08%, tg: 0.54 ± 0.11%, P=0.02). Moreover, NF-E2 tg mice contain significantly elevated numbers of both CMP and MEP (common myeloid progenitors; CMP per femur: wt: 46,600±5,300, tg: 63,200±5,100, p =0.02; megakaryocyte-erythroid progenitors; MEP per femur: wt: 58,600 ± 13,700, tg: 115,600 ± 28,800, P=0.02). In order to determine whether enhanced proliferation of HSCs contributes to their increase, cell cycle analysis of the various stem and progenitor compartments is being conducted and the data will be presented. *Summary.* In a murine model, NF-E2 overexpression causes expansion of the hematopoietic stem and progenitor compartments, specifically the most primitive stem cells, LT-HSC, as well as the megakaryocytic and erythroid precursors, the MEPs. These data strongly suggests that the NF-E2 overexpression observed in CD34+ cells of MPN patients contributes to the increase in HSCs present in these disorders.

IDH mutations in acute myeloid leukemia

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ACQUIRED MUTATIONS IN THE GENES ENCODING IDH1 AND IDH2 BOTH ARE RECURRENT ABERRATIONS IN ACUTE MYELOID LEUKEMIA (AML): PREVALENCE AND PROGNOSTIC VALUE

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Introduction. Somatic mutations in the genes encoding the isocitrate dehydrogenases IDH1 and IDH2 were revealed in more than 70% of astrocytomas, oligodendrogliomas and glioblastomas. Mutations in *IDH1* and *IDH2* affects the arginines on position 132 of IDH1 and position 172 of IDH2, resulting in disturbed function of both isocitrate dehydrogenases, as demonstrated by impaired production of NADPH. Recently, acquired mutations in the gene encoding IDH1 were identified in 8% and 5.5% of newly diagnosed AML cases. Overall, the *IDH1* mutation status did not suggest a relationship with overall survival. In this study, we determined the frequencies of both *IDH1* and *IDH2* mutations in cohorts of AML, ALL, CML and JAK2 V617F MPN. In a cohort of 893 cases of AML, we investigated their distribution in relationship with cytogenetic and molecular risk categories as well as recurrent gene mutations commonly apparent in AML and we evaluated the impact of *IDH* mutations on treatment outcome. *Methods. IDH1* and *IDH2* mutations in AML, RAEB, ALL, CML and JAK2 V617F MPN were determined by cDNA amplifications. All IDH1 and IDH2 RT-PCR products were subjected to denaturing high performance liquid chromatography (dHPLC) analyses using a WAVE system. PCR products showing aberrant dHPLC profiles were purified using the Multiscreen-PCR 96-well system followed by direct sequencing. We validated this strategy using 350 cases of *de novo* AML that were previously analyzed using PCR on genomic DNA followed by direct sequencing. The relation between *IDH* mutations and various patient characteristics were determined by the Student's t-test equal variances not assumed (continuous variables) and the Fisher's exact test (categorical variables). Distribution estimations and survival distributions of overall survival (OS) and event-free survival (EFS) were calculated by the Kaplan-Meier method and the log-rank test. *Results.* A RT-PCR/dHPLC screen of 893 newly diagnosed AMLs followed by direct sequencing, identified *IDH1* mutations in 54 AML cases (6.0%) and IDH2 mutations in 23 cases (2.6%). A total of 77 (8.6%) mutations in either *IDH1* or *IDH2* were apparent in 76 cases. No *IDH* mutations were found in the ALL and CML cohorts. *IDH1* and *IDH2* mutations were significantly more frequently present among cytogenetically defined intermediate risk AML, as well as cytogenetically normal AML. In addition, *IDH* mutations appear to be significantly associated with $NPM1^{mutant}$. To investigate the prognostic value of *IDH* mutations, 893 AML patients were considered for survival analysis in various AML subtypes. There was a trend for worse EFS for IDH^{mutant} patients within the AML subtype $NPM1^{wild\text{-}type}$ (P=0.04). However, among the $FLT3^{wild\text{-}type}/NPM1^{wild\text{-}type}$ AML subtype (n=15 cases) the presence of *IDH1* mutations predicted for both significantly reduced OS (P<0.032) and EFS (P=0.005). *Conclusions.* Acquired *IDH* gene mutations, i.e., not only in *IDH1* but also *IDH2*, are common abnormalities in AML. Together, *IDH1* and *IDH2* mutations account for a considerable frequency of approximately 9% in adult AML. *IDH* gene mutations are associated with normal karyotypes and *NPM1* mutations. *IDH1* mutations appear to correlate with significantly inferior outcome in patients $FLT3^{wild\text{-}type}/NPM1^{wild\text{-}type}$ AML, but requires confirmation in future studies.

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PROGNOSTIC IMPACT OF ISOCITRATE DEHYDROGENASE ENZYME ISOFORMS 1 (IDH1) AND 2 (IDH2) MUTATIONS IN ACUTE MYELOID LEUKEMIA. A STUDY BY THE ACUTE LEUKEMIA FRENCH ASSOCIATION (ALFA) GROUP

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Background. Recently, whole-genome sequencing in acute myeloid leukemia (AML) identified recurrent *IDH1* mutations (*IDH1m*), previously reported to be involved in gliomas as well as *IDH2* mutations (*IDH2m*). IDH1 is a cytosolic protein that catalyzes the oxidative carboxylation of isocitrate to α-ketoglucarate (α-KG), leading to NADPH production in Krebs cycle. Isocitrate dehydrogenase 2 (IDH2) gene encodes a mitochondrial protein homologous to IDH1 that also catalyses isocitrate carboxylation. Mutations affecting analogous amino acids R132 IDH1 and R172 IDH2 that are located in the active site of the enzymes, lead to inhibition of wild-type IDH1 and IDH2 activity and catalyse α-KG to R-2-hydroxyglutarate (R2H), a metabolite that is suspected to trigger brain tumors. In AML patients, IDH1m have been shown to be associated with FAB M1 subtype and CN-AML The prognosis of both IDH1m and IDH2m in AML remains unclear. *Methods.* The prevalence and the prognostic impact of R132 IDH1 and R172 IDH2 mutations were evaluated in a cohort of 520 adults with AML homogeneously treated in the French ALFA-9801 and -9802 trials. The screening of *IDH1m* and *IDH2m* was performed by direct sequencing. *Results.* The prevalence of *IDH1m* and *IDH2m* was respectively 9.6% and 3.0%, mostly associated with normal cytogenetics (CN). Among the 50 patients harbouring *IDH1m*, we found 22 R132H (44%), 21 R132C (42%), 6 R132S (12%) and 1 R132L mutation (2%), contrarily to what was reported in gliomas where R132 IDH1 is found mutated to histidine in more than 88% of the cases. All cases with *IDH1m* or *IDH2m* were heterozygous for the mutation. Patients with CN-AML and *IDH1m* or *IDH2m* were slightly older (respective median age: 54y and 57y *vs.* 48y, P=.07 and P=.09). In those patients, *IDH1m* was associated with *NPM1m* (P=.008) but exclusive of *CEBPAm* (P=.03). The rate of *FLT3*-ITD was similar in *IDH1m* and *IDH1wt* patients (18% *vs.* 20%, P=.99). In contrary, no other mutations were detected in IDH2m patients. In CN-AML patients, *IDH1m* were found in 19% of favorable genotype ((*NPM1m* or *CEBPAm*) without *FLT3*-ITD) and were associated with a higher risk of relapse (5y-RR: 71% for *IDH1m vs.* 38%, P=.01) and a trend to shorter overall survival (5y-OS: 45% for *IDH1m vs.* 64%, P=.10). Favorable genotype in CN-AML could thus be defined by the association of *NPM1m* or *CEBPAm* with neither *FLT3*-ITD nor *IDH1m*. Patients with this favourable profile (N=62) had a lower RR (5y-RR: 39% *vs.* 76%, P<.0001) and a better OS (5y-OS: 68% *vs.* 29%, P<.0001). In *IDH2m* CN-AML patients (N=7), we observed a higher risk of induction failure, a higher RR (5y-RR: 100% for *IDH2m vs.* 60%, P=.01) and a shorter OS (5y-OS: 0% for *IDH2m vs.* 46%, P=.005). In multivariate analysis, age, WBC count, the 4-gene favorable genotype and *IDH2m* were independently associated with a higher RR and a shorter OS. *Conclusion.* Contrarily to what is reported in gliomas, *IDH1m* and *IDH2m* are associated with a poor prognosis in AML. Screening of *IDH1m* could help to identify high risk patients within the subset of CN-AML with a favorable genotype.

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IDH1 AND IDH2 GENE MUTATIONS IDENTIFY NOVEL MOLECULAR SUBSETS WITHIN DE NOVO CYTOGENETICALLY NORMAL ACUTE MYELOID LEUKEMIA (CN-AML): A CANCER AND LEUKEMIA GROUP B (CALGB) STUDY

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Background. Recent studies revealed that mutations in the *IDH1* and *IDH2* genes, which encode isoforms of the NADP(+)-dependent isocitrate dehydrogenases, are recurrent in brain tumors and are associated with favorable outcome. Importantly, *IDH1*, but not *IDH2*, mutations were also discovered in AML. The presence of *IDH1* mutations was associated with CN-AML, and found to confer adverse prognosis in a subset of CN-AML patients without mutations in the *NPM1* gene (Mardis *et al*. N Engl J Med 361:1058-1066, 2009). However, larger studies of similarly treated patients are necessary to assess the frequencies and prognostic significance of mutations in both the *IDH1* and *IDH2* genes in CN-AML. *Aims.* To evaluate the frequency and analyze associations with prognostic markers and outcome of mutations in the *IDH1* and *IDH2* genes in adults with *de novo* CN-AML. *Methods.* Diagnostic marrow or blood samples from 358 patients treated with age-adapted intensive chemotherapy regimens on CALGB first-line protocols were analyzed for *IDH1* and *IDH2* mutations by DNA PCR amplification/sequencing. *FLT3*, *NPM1*, *CEBPA*, *WT1* and *MLL* mutational analyses and gene- and microRNA-expression profiling were performed centrally. *Results. IDH* mutations were found in 33% of the patients. *IDH1* mutations were detected in 49 patients (14%; 47 with R132). *IDH2* mutations, previously unreported in AML, were detected in 69 patients (19%; 13 with R172 and 56 with R140). R172 *IDH2* mutations were mutually exclusive with all prognostic mutations analyzed. Younger (<60 years) molecular low-risk (*NPM1*-mutated/*FLT3*-ITD-negative) *IDH1*-mutated patients had shorter disease-free survival than molecular low-risk, *IDH1/IDH2*-wild-type (wt) patients (P=.046). R172 IDH2-mutated patients had lower complete remission rates than *IDH1/IDH2*wt patients (P=.007). Distinctive microarray gene- and microRNA-expression signatures accurately predicted R172 *IDH2* mutation status (95.5% and 93.4% cross-validated prediction accuracy for gene- and microRNA-expression signatures, respectively). The highest expressed gene and microRNAs in R172 *IDH2*-mutated patients compared with the *IDH1/IDH2*wt patients were, respectively, APP, previously associated with complex karyotype AML, and *miR-1* and *miR-133*, involved in embryonal stem-cell differentiation. *Summary. IDH1* and *IDH2* mutations are recurrent in CN-AML and impact unfavorably on outcome. *IDH1* mutations are associated with shorter disease-free survival in younger patients with molecular low-risk CN-AML. R172 *IDH2* mutations are mutually exclusive with other known prognostic mutations and denote a novel subset of older CN-AML characterized by resistance to induction chemotherapy, whereas R140 *IDH2* mutations do not appear to confer prognostic significance. By deriving gene- and microRNA-expression signatures, we uncovered intriguing features in R172 *IDH2*-mutated patients that may lead to better understanding of the biologic role of this mutation, and to designing of novel therapies targeting aberrant IDH-driven activation of metabolic pathways.

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IDH1 AND IDH2 GENE MUTATIONS ARE FREQUENT MOLECULAR LESIONS IN ACUTE MYELOID LEUKEMIA (AML) AND CONFER ADVERSE PROGNOSIS IN CYTOGENETICALLY NORMAL AML WITH NPM1 MUTATION WITHOUT FLT3-ITD

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Background. Isocitrate dehydrogenase 1 (*IDH1*) and 2 (*IDH2*) genes encode metabolic enzymes that convert isocitrate into α-ketoglutarate (α-KG). *IDH1* and *IDH2* (hereafter commonly referred to as *IDH*) mutations have been described as recurrent molecular lesions in gliomas and in acute myeloid leukemia (AML). *IDH* mutations cluster in exon 4 and result in a neomorphic enzymatic activity to convert α-KG into 2-hydroxyglutarate. While in gliomas IDH mutations have been associated with favorable outcome, their prognostic relevance in AML still remains to be elucidated. *Aims.* The objectives of this study were to assess the frequencies of *IDH* mutations and to explore their associations with clinical, cytogenetic and molecular characteristics as well as their prognostic relevance in a large cohort of AML patients. *Methods.* Pretreatment bone marrow or blood specimens from 805 patients with AML aged <60 years and entered on one of two AMSLG treatment trials [AML HD98A (n=734) and APL HD95 (n=71)] were studied for the presence of *IDH* mutations; exon 4 of *IDH1* and *IDH2*, respectively, was amplified by PCR and the amplicons were analyzed using a combination of denaturing high-performance liquid chromatography and DNA sequencing. The patients were also assessed for *FLT3* (ITD and TKD), *NPM1*, *CEBPA* and *MLL*-PTD mutations by standard PCR techniques. *Results. IDH* mutations were found in 129 (16.0%) patients [*IDH1* in 61 (7.6%), *IDH2* in 70 (8.7%)]; two cases had both *IDH1* and *IDH2* mutations. All but one *IDH1* mutation caused substitutions of residue R132. *IDH2* mutations resulted in changes of residues R140 (n=48) or R172 (n=22). *IDH* mutations were associated with cytogenetics; 67% of the patients with IDH mutations had a normal karyotype, 22 (19%) cases had other intermediate-risk cytogenetics, and 15 (13%) cases had adverse-risk cytogenetics. Only 1 one of 71 APL patients, and no patients with core-binding factor AML harbored *IDH* mutations. In AML with adverse-risk cytogenetics, *IDH* mutations were mainly represented by *IDH2* mutations (13 of 15; $IDH2^{R140Q}$, n=6; $IDH2^{R172K}$, n=7). With regard to clinical and molecular features, *IDH* mutations were associated with older age (P<.001; effect conferred by *IDH2* mutations only), higher platelets (P<.001), cytogenetically normal (CN)-AML (P<.001), and *NPM1* mutations, in particular with the genotype "mutated *NPM1* without *FLT3*-ITD"(P<.001). In univariable and multivariable analyses, IDH mutations did not impact response to induction; the same was true when patients with APL were excluded or the analysis was restricted to CN-AML patients. In univariate analyses, *IDH* mutations adversely impacted overall survival (OS; P=.03) only in CN-AML with mutated *NPM1* without *FLT3*-ITD (5-year OS rates: 41% v 65% for patients with and without *IDH* mutations, respectively); however, in multivariate analyses, *IDH* mutations were a poor prognostic factor for relapse-free survival (P=.03) and OS (P=.007) in this patient subset. *Summary/Conclusions. IDH* mutations are recurring genetic changes in AML, in particular in *NPM1*-mutated cases and constitute a poor prognostic factor among CN-AML with mutated *NPM1* without *FLT3*-ITD allowing to further refine the risk stratification of this favorable AML subset.

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IDH1 MUTATIONS ARE DETECTED IN 6.6% OF ALL AML AND ARE STRONGLY ASSOCIATED WITH INTERMEDIATE RISK KARYOTYPE AND UNFAVOURABLE PROGNOSIS: A STUDY OF 1414 PATIENTS

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Introduction. IDH1 is the gene coding for the soluble isocitrate dehydrogenease 1 ($NADP^+$), which catalyzes the oxidative decarboxylation of isocitrate to 2-oxoglutarate. The gene has been shown to be frequently mutated in high-grade gliomas at residue p.R132, which is located in the substrate binding site of IDH1. Recently a next generation sequencing project found IDH1 mutated in AML with normal karyotype (Mardis *et al.,* NEJM, 2009). *Aim.* To further evaluate the importance of IDH1R132 (IDH1mut) in AML we have analyzed a cohort of 1414 comprehensively characterized AML cases. *Methods.* The respective base exchange was analysed by a LightCycler based melting curve assay with subsequent sequencing of the positive samples. *Results.* The cohort was comprised of 1170 *de novo* AML (82.7%), 157 AML following MDS (s-AML,11.1%) and 87 AML after previous treatment of different malignancies (t-AML, 6.2%). Median age was 63 years (range (17.1-93.3 years). Karyotype was available in all cases: 673 had a normal karyotype (NK) AML, and 741 had chromosomal aberrations (t(15;17): n=88; inv(16): n=57, t(8;21): n=83, t(11q23): n=31, t(6;9): n=5, inv(3): n=15; -7: n=32, +8: n=30, +13: n=12, complex aberrant: n=288, others: n=1806). Overall, in 93 pts IDH1 p.R132 mutations were detected (6.6%). Five different amino acid exchanges were observed: R132C (n=51), R132L (n=22), R132 H (n=6), R132G (n=9), and R132S (n=5). With respect to history of the patient IDH1mut were found in 80/1170 of *de novo* AML (6.8%), 11/157 (7.0%) of s-AML, and 2/87 (2.3%) of t-AML, respectively (n.s.). Significantly more females (58/668, 8.7%) than males (35/741; 4.7%) revealed the IDH1mut (P=0.003). Age was slightly higher in the mutated cases (66.0 *vs.* 62.6 years, P=0.075). There was a clear association to the intermediate risk karyotype group (10.4%, P<0.001). Although IDH1R132 mutations were detected incidentally together with all other molecular markers there were strong associations with NPM1 mutations (14.2 *vs.* 5.4% in NPM1wt, P<0.001) and MLL-PTD (18.2% *vs.* 7.0% in MLLwt, P=0.020). There were further strong associations to AML without maturation/FAB M1 (P<0.001) and an immature immunophenotype. Prognosis was adversely affected by IDH1 mutations with trend for shorter OS (533 days *vs.* median not reached; P=0.110) and a shorter EFS (365 *vs.* 523 days; P<0.003). Although IDH1 mutations were detected preferentially in NPM1 mutated AML, an adverse impact of IDH1 mutations was detected only in the NPM1wt group (median 283 days for IDH1mut *vs.* 668 days for IDH1wt, P=0.044). In the age group <60 years IDH1 mutations were of independent prognostic relevance (P=0.028). Conclusions, these data show that IDH1R132 is a further important marker in AML that is useful for further characterization and prognostication. In addition, IDH1 mutations seem to be a new class of mutation probably complementing with the classical type 1 and type 2 mutations.

Chronic myeloid leukemia - Clinical 1

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MAJOR MOLECULAR RESPONSE RATE AT ONE YEAR IS HIGHER IF PEGYLATED INTERFERON ALPHA-2B IS ADDED TO IMATINIB IN NON-HR CHRONIC MYELOID LEUKEMIA PATIENTS IN IMATINIB INDUCED COMPLETE HEMATOLOGICAL REMISSION

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Background. Imatinib mesylate (IM) 400 mg once daily (OD) is the current standard first-line therapy for CML. Several biological and clinical observations suggest that combining IM with interferon (IFN) alpha may improve the outcome of treatment. *Aim.* To compare the effects of standard-dose IM to combination of IM and IFN alpha in newly diagnosed chronic phase CML patients with an intermediate or low Sokal risk score in IM induced complete hematological remission (CHR). The primary objective was to compare the major molecular response (MMR) rate after 12 months between the treatment arms (intention-to-treat analysis). *Patients and therapies.* In a Nordic CML Study Group (NCMLSG: Denmark, Finland, Norway and Sweden) and Israel multicenter study we randomized (after informed consent was obtained) 112 newly diagnosed CML patients in CHR following 3 months of IM 400 mg OD induction therapy. The study arms were IM (Glivec, Novartis) and the combination of IM and IFN alpha-2b (PegIntron, Schering-Plough) (Peg-IFN). IM dose was fixed at 400 mg OD. The starting dose of Peg-IFN was initially 50 μg once weekly, but rather soon due to side effects reduced to 30 μg once weekly. Depending on tolerability it could then be escalated to 50 or reduced down to 15 _g once weekly. Molecular response was evaluated by blood RQ-PCR for BCR-ABL1 and expressed on the international scale (IS). *Results.* MMR rate at 52 weeks was significantly higher (P=0.002) for the IM+Peg-IFN arm (82%) compared to the IM only arm (54%) (intention to treat analysis). No unpredictable complications or adverse events were reported. Four patients (8%) discontinued IM in the IM arm (progression to ABP, protocol violation, loss of CCgR, biochemical AE). In the combination arm 4 patients (8%) discontinued IM (progression to ABP, no CgR, nonhematological AEs) and 30 (58%) discontinued Peg-IFN (refusal, hematological and non-hematological AEs,). The MMR rate increased with the duration of Peg-IFN treatment (<12 weeks MMR rate 67%, >38 weeks MMR rate 91%). *Conclusions.* In newly diagnosed CML patients with an intermediate or low Sokal risk score and in IM induced CHR, the MMR rate at 52 weeks was significantly higher (P=0.002) for the IM+Peg-IFN arm (82%) compared to the IM only arm (54%) (intention to treat analysis). There were no unpredictable complications or adverse events reported.

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TREATMENT OPTIMIZATION BY HIGH DOSE IMATINIB: RANDOMIZED COMPARISON OF IMATINIB 800 MG VS. IMATINIB 400 MG *VS.* IMATINIB 400 MG + IFN IN NEWLY DIAGNOSED BCR-ABL POSITIVE CHRONIC PHASE (CP) CML WITH REGARD TO MMR AT MONTH 12. THE GERMAN CML-STUDY IV

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Initial imatinib therapy has never been optimized. Faster induction of molecular and cytogenetic remissions by imatinib 800 mg has been reported. The German CML Study Group therefore compared imatinib 800 mg (IM 800) with standard dose imatinib ± IFN (IM 400, IM+IFN) in newly diagnosed, not pretreated CP-CML with regard to major molecular response (MMR) at 12 months in a randomized clinical trial. By April 30, 2009, 1022 patients were randomized. Comparison was for molecular and cytogenetic remissions, overall (OS) and progression free (PFS) survival and toxicity. 1016 patients were evaluable at baseline, 954 for survival analysis (305 for IM 400, 310 for IM 800, 339 for IM+IFN), 845 for cytogenetic and 871 for molecular remissions. Median follow-up was 28 months in the IM 800 arm and 47 (44) months in the imatinib 400±IFN arms, respectively. The difference is due to a later start of the IM 800 arm. The median daily doses of imatinib were 646 mg (209- 800 mg) in the IM 800 arm and 400 mg (184- 720 mg) in the IM 400±IFN arms. Of 218 patients receiving IM 800 and evaluable for dosage at 12 months, 100 (45.9%) received more than 700 mg, 27 (12.4%) 601-700 mg, 37 (17.0%) 501-600 mg, 48 (22.0%) 401-500 mg and only 6 (2.8%) 400 mg/day or less. The cumulative incidences of achieving complete cytogenetic remission (CCR) and MMR with IM 400, IM 800 and IM+IFN at 6, 12, 18 and 24 months after start of treatment and the differences of CCR and MMR rates between treatment arms are summarized in the table. MMR at 12 months was reached in more patients with IM 800 than with IM 400 (P=0.0001) or IM+IFN (P=0.0009). Optimal molecular response (<0.01% BCR-ABL according to the international scale) was reached with IM 800 after a median of 31.3 *vs.* 47.5 and 42.5 months with IM 400±IFN, respectively. Also CCR was reached faster with IM 800 (P<0.01). The more rapid achievement of MMR with IM 800 was observed in low and intermediate risk, but not in high risk patients. At the time of this evaluation, OS (92%) and PFS (88%) at 5 years showed no difference between treatment arms. After dose adjustments IM 800 was well tolerated. Hematologic (thrombocytopenia 7% *vs.* 4%) and non-hematologic adverse events (AE) (gastrointestinal 35% *vs.* 15-24% and edema 29% *vs.* 16-19%) were more frequent with IM 800, fatigue (14% *vs.* 7-13%) and neurological problems (15% *vs.* 6-7%) more frequent with IM+IFN. Grade III/IV AEs as analyzed over the total time of therapy were rare and did not differ between treatment arms. In conclusion, these data show a significantly faster achievement of MMR and CCR with IM 800 as compared to IM 400±IFN. The data indicate that the optimal imatinib dose in CP may be higher than 400 mg per day. Longer observation will determine whether or not this more rapid achievement of MMR and CCR will translate into better survival.

Table.

Time after start of treatment	Cumulative incidences of achieving a									
	CCR(%)					MMR(%)				
	IM400 n=273	Δ	IM800 n=268	Δ	IM+IFN n=304	IM400 n=279	Δ	IM800 n=283	Δ	IM+IFN n=309
6 mo	21.5	*12.0*	33.5	*15.1*	18.4	6.1	*11.3*	17.4	*11.2*	6.2
12 mo	49.8	*13.4*	63.2	*13.7*	49.5	31.1	*24.7*	55.8	*22.8*	33.0
18 mo	66.6	*7.2*	73.8	*3.8*	70.0	51.5	*18.7*	70.2	*15.5*	54.7
24 mo	74.5	*8.5*	83.0	*6.6*	76.4	64.2	*13.3*	77.5	*14.7*	62.8

Δ: Difference between IM 800 and IM 400 or IM 800 and IM+IFN, respectively.

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SIGNIFICANT IMPROVEMENT OF MOLECULAR RESPONSES WITH PEGYLATED FORM OF INTERFERON A2A IN COMBINATION WITH IMATINIB (IM) IN CHRONIC MYELOID LEUKAEMIA (CML) PATIENTS (PTS) REPORT OF A PHASE III TRIAL

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Background. IM 400 mg daily is the front-line treatment of CP CML, but provides only 50% major molecular responses (MMR) at 18 months (Mo). *Aims.* we designed a phase III randomized multicenter open-label prospective trial comparing IM 400 mg/d (n=159) with 3 experimental arms: IM 600 mg/d (n=160), IM 400 mg/d combined to s/c cytarabine (Ara-C), (20 mg/m^2/d, d15-28 of 28-day cycles)(n=158) and IM 400 mg/d combined to s/c Peg-IFN2a (90 µg/wk) (n=159). *Methods.* Pts were allocated at a 1.1.1.1 ratio, stratified by Sokal risk groups, molecular assessments being calculated according to the international standardized scale (IS). The purpose of this trial ,conducted according to 2 part, was to first determine whether higher doses of IM or combining IM with interferon or Ara-C would result in higher rates of molecular responses and if so, in better survival. During the part 1, the increased dose of IM or a combination regimen would be considered as promising at 1 year, if it increased the 4 log reduction response rate by at least 20 percentage points, e.g. from 15% to 35%. A planned interim analysis of 636 pts based on an optimal molecular response (OMR = BCR-ABL/ABL ratio ≤0.01) (α=0.85%, β=10%) at 1 year has suggested the superiority of the combination of Peg-IFN2a and imatinib (ASH 2008). We now report the 24 months update of this planned interim analysis. *Results.* Pts of the part 1 were recruited between 9/2003 and 10/2007, median age 51 yrs (18-82), 62% males; Sokal score was low 37%, intermediate 39% and high risk 24%. Median follow-up was 48 Mo. (range 24-73) for alive patients. Rates of MMR at 24 months (ITT) were for IM+Peg-IFN2a , IM 400 mg, IM 600 mg, and IM + Ara-C 71%, 48%, 62% and 63% (P value PegIFN *vs.* IM 400 mg:P<.0001); the corresponding number for OMR were 46%, 26%, 34%, and 34% (P=.0006); for undectable molecular residual disease 22%, 11%, 11%, 10% (P=.0028). During the first year of treatment the median dose of IM was 400 mg for the 3 arms including IM 400 and 590 mg for IM 600; the median dose for Peg IFN2a was 54 µg per week (range11-166) and was 24mg per day (range 10-40) for Ara-C. Overall, 45% of the pts discontinued Peg-IFN2a during the first 12 months. Of interest, duration of treatment with Peg-IFN2a had an impact on responses. In pts who have been treated less than 4 months as compare to more than 12 months, rate of MMR, OMR and UMRD increased from 48-82%, 23-49% and 8-20% respectively. Grade 3/4 neutropenia and/or thrombocytopenia occurred more frequently with the combination arms; the proportion of patients with grade 3 or 4 adverse events was relatively low in all arms. *Conclusions.* Based on these results accrual was stopped into the IM 600 mg and IM 400 mg

+ Ara-C arms and the trial is currently recruiting with IM 400 mg as control arm and the combination IM400 mg + Peg-IFN2a as best experimental arm.

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CONTINUED SUPERIORITY OF NILOTINIB VS IMATINIB IN PATIENTS WITH NEWLY DIAGNOSED CHRONIC MYELOID LEUKEMIA IN CHRONIC PHASE (CML-CP): ENESTND BEYOND 1 YEAR

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Background. Results of ENESTnd demonstrated superior efficacy of nilotinib 300 and 400 mg bid over imatinib, including significantly higher rates of major molecular response (MMR) and complete cytogenetic response (CCyR). *Aims.* Here, we present new data including molecular and cytogenetic responses in evaluable patients and event-free survival (EFS). *Methods.* 846 patients with CML-CP were randomized to nilotinib 300 mg bid (n=282), nilotinib 400 mg bid (n=281), and imatinib 400 mg qd (n=283). Primary endpoint was MMR (≤ 0.1% BCR-ABLIS) rate at 12 months. EFS events defined as death due to any cause, progression to advanced phase or blast crisis, loss of CCyR, loss of partial CyR, or loss of complete hematologic response. *Results.* Rates of MMR at 12 months were superior for nilotinib 300 and 400 mg bid compared with imatinib (Table). Superior rates of MMR at 12 months were observed in both nilotinib arms compared with the imatinib arm across all Sokal risk groups (Table). Higher rates of MMR were also observed in both nilotinib arms at 15 months in evaluable patients, with 57% achieving MMR in both nilotinib arms compared with 33% in the imatinib arm. Molecular responses were deeper on nilotinib, with more patients achieving BCR-ABL (IS) transcript level reductions of ≤ 0.01% and ≤ 0.0032% compared with imatinib. CCyR rates by 12 months were higher in evaluable patients treated with nilotinib 300 mg bid (93%) and nilotinib 400 mg bid (93%) compared with imatinib (76%). More patients experienced cytogenetic treatment failure (ELN 2006) on imatinib (8%) compared with nilotinib 300 mg bid (1%) or 400 mg bid (2%). Progression to advanced disease was significantly lower for nilotinib at both doses compared with imatinib (Table).

Table.

	Nilotinib 300 mg bid (n = 282)	Nilotinib 400 mg bid (n = 281)	Imatinib 400 mg qd (n = 283)
MMR, %			
At 12 months (ITT)	44 $P < .0001$*	43 $P < .0001$*	22
At 12 months (evaluable patients) (n=242, 240, 235)	51	50	27
At 15 months (evaluable patients) (n=154, 155, 145)	57	57	33
MMR at 12 months by Sokal risk group (ITT), %			
Low	41 $P = .0238$†	53 $P < .0001$†	26
Intermediate	51 $P < .0001$†	40 $P = .0085$†	23
High	41 $P = .0008$†	32 $P = .0252$†	17
CCyR, %			
By 12 months (ITT)	80 $P < .0001$*	78 $P = .0005$*	65
By 12 months (evaluable patients) (n=244, 236, 242)	93	93	76
K-M estimated rate at 12 months			
EFS, %	97.6 $P = .0898$‡	99.6 $P = .0012$‡	95.7
Progression to AP/BC, %	0.7 $P = .0095$‡	0.4 $P = .0037$‡	3.5

* CMH test stratified by Sokal vs imatinib

† Chi-square test

‡ Log-rank test stratified by Sokal vs imatinib for EFS and time to AP/BC

Estimated 12-month rate of EFS was higher in nilotinib 300 mg bid (97.6%) and nilotinib 400 mg bid (99.6%) compared with imatinib (95.7%). No patient who achieved MMR progressed. Of 11 patients who progressed on imatinib, 10 had secondary resistance, and 6 had mutations. Both drugs were well-tolerated. Discontinuations due to adverse events or laboratory abnormalities were lowest for nilotinib 300 mg bid (7%). Minimum 16-month follow-up data will be presented (maximum, 29 months). *Conclusions.* Nilotinib induced higher response rates compared with imatinib with longer follow-up. This superior efficacy resulted in significantly improved progression rates. These data support the potential of nilotinib to become the new standard of care in patients with newly diagnosed CML.

1114

NILOTINIB 400 MG BID IN EARLY CHRONIC PHASE PH+ CHRONIC MYELOID LEUKEMIA: RESULTS AT 2 YEARS OF A PHASE II TRIAL

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Background. Imatinib (IM) 400 mg daily is the standard treatment for Ph^+ chronic myeloid leukemia (CML) in early chronic phase (ECP). According to the IRIS trial, the rate of progression to advanced phase and the rate of any event (loss of complete hematologic response, loss of major cytogenetic response, progression or death) are higher during the first period of treatment: particularly, during the first 2 years they were 10.8% and 4.3%, respectively. Moreover, only 3% of patients who achieved a complete cytogenetic response (CCgR) progressed to advanced phase, all but 1 within 2 years of achieving CCgR. In a phase III trial (ENESTnd, ASH Meeting 2009), nilotinib at both 300 mg and 400 mg bid induced significantly higher and faster rates of major molecular response (MMR) and CCgR compared with imatinib 400 mg daily; the median observation time was 14 months. *Aims.* To investigate the dynamics and deepness of molecular response, the stability of responses, the progression-free and event-free survival, in ECP Ph-pos CML patients treated with nilotinib 400 mg BID, with a minimum follow-up of 24 months (results at 12 months have been previously published , Rosti *et al.*, Blood 2009). *Methods.* A multicentric phase II trial was conducted by the GIMEMA CML Working Party (ClinicalTrials.gov.NCT00481052). The molecular response was studied serially by Q-PCR (MMR, ≤ 0.1% according to the International Scale). By March, 2010 all the patients will complete 24 months on treatment. *Results.* 73 patients have been enrolled; median age 51 years (range 18-83), 45% low, 41% intermediate and 14% high Sokal risk. Median follow-up is currently 724 days. The cumulative CCgR rate within 12 months was 100%. The CCgR rate was 78% at 3 months and 96% at 6,12 and 18 months. The median duration of CCgR was 18 months. The rates of MMR at 1, 2, 3, 6, 12, 15, 18 and 21 months were 3%, 21%, 52%, 66%, 85%, 87%, 87% and 87%, respectively. At 12 and 18 months, 7% and 16% of patients tested negative with nested PCR, respectively. One patient only progressed at 6 months to advanced phase (ABP) with T315I mutation. Adverse events (AEs) were mostly grade 1 and 2 and manageable with appropriate dose adaptations; 2 pts went off treatment after 9 and 15 months due to recurrent episodes of amylase and/or lipase increase (no pancreatitis). *Conclusions.* At 2 years, an increasing number of patients achieved transcript-undetectable status; responses to nilotinib, either CCgR and MMR, remain stable. No progressions to advanced phase have been observed during the second year. *Acknowledgements.* European LeukemiaNet, COFIN, Bologna University, BolognAIL.

Acute myeloid leukemia - Experimental therapeutics

1115

PRELIMINARY RESULTS OF A PHASE II TRIAL OF LOW-DOSE CLOFARABINE IN COMBINATION WITH THE MTOR INHIBITOR TEMSIROLIMUS AS FIRST SALVAGE THERAPY FOR ELDERLY PATIENTS WITH ACUTE MYELOID LEUKEMIA (GIMEMA AML-1107)

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Background. Elderly patients with refractory/relapsed AML have a dismal prognosis and new treatments are needed for these patients. Constitutive activation of the PI3K/AKT/mTOR pathway regulates the growth and survival of AML cells and may contribute to chemoresistance. mTOR inhibition with rapamycin analogs has been associated with responses in AML and may enhance the sensitivity of leukemic cells to cytotoxics. Clofarabine is a nucleoside analog with activity in elderly AML. Based on this, we designed a multicenter phase II study of low-dose Clofarabine in combination with Temsirolimus in elderly patients with advanced AML. *Aims.* To assess efficacy and toxicity of this regimen when given as first salvage therapy; primary endpoint is the rate of CR+CRi. Enrollment is proceeding according to a 2-stage design with a minimum of 19 pts in stage 1 and expansion to a total of 54 evaluable pts if at least 5 CR/CRi were observed in the initial cohort. In addition, correlative biomarker studies will examine if leukemia mTOR pathway status predicts clinical response. *Methods.* Eligible were pts age ≥ 60 yrs with first relapse or primary refractory AML. Induction consisted of one course of Clofarabine 20 mg/m^2 iv d 1-5 and Temsirolimus 25 mg (flat dose) iv d 1, 8 and 15; a second course was allowed in pts achieving PR. Pts achieving CR/CRi receive up to 12 monthly courses of maintenance with Temsirolimus (25 mg iv d 1 and 8 per course). *Results.* Between 04/09 and 10/09, 21 pts were enrolled and all are evaluable for response. Demographics: 52% males; median age 69 yrs (range 60-77); primary refractory AML 24%, first relapse AML 76% (duration of CR1 < 12 mos 75%, ≥ 12 mos 25%); cytogenetics: intermediate 67%, adverse 14%, and unknown 19%. Eight pts achieved a CR (3) or CRi (5) for an ORR of 38%. The 30-day all-cause mortality rate was 14% (2 pts died from infection, 1 pt died of progressive AML+pneumonia). CR/CRi was achieved in 1/5 pts with primary refractory AML (20%), and 7/16 in first relapse (44%). In particular, responses were noted in 6/12 (50%) pts with short CR1 (< 12 mos), and in 1/4 (25%) pts with longer CR1. CR/CRi rates by cytogenetics and age are: intermediate 4/14 (29%), adverse 0/3, unknown 4/4 (100%); < 70 yrs 4/12 (33%), ≥ 70 yrs 4/9 (44%). Median duration of remission is 3 mos; 5 pts have relapsed to date and 3 (1 CR, 2 CRi) remain on Temsirolimus maintenance at 5+, 6+ and 7+ mos from remission. Median follow-up is 5 mos and 11 pts remain alive at 6-9+ mos. Besides cytopenias, the most common CTCAE grade 3/4 were infection (67%), febrile neutropenia (43%), diarrhea (10%), nausea/vomiting (10%), and transaminase elevation (10%). Median times to recovery of neutrophils to 500/cmm and platelets to 50,000/cmm in responders were 28 and 32 days, respectively. *Conclusion.* These preliminary results suggest that the combination of low-dose Clofarabine and Temsirolimus has encouraging clinical activity in this difficult-to-treat patient population, with a manageable safety profile. Accrual and correlative biomarker analyses continue.

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PP2A IMPAIRED ACTIVITY IS A COMMON EVENT IN ACUTE MYELOID LEUKEMIA, AND ACTIVATION BY FORSKOLIN TREATMENT INDUCES A POTENT ANTILEUKEMIC EFFECT

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Protein phosphatase 2A (PP2A) is a major phosphatase and tumor suppressor that has been described as a potential therapeutic target for chronic myeloid leukemia, Philadelphia-chromosome positive acute lymphoblastic leukemia, and B-cell chronic lymphocytic leukemia. Moreover, it has been described a reduced PP2A activity in a small series of 13 AML patients with complex karyotype (n=7), in comparison with intermediate-risk karyotype (n=6). We have previously reported that SETBP1 overexpression is a recurrent event in AML (27%) that impairs PP2A activity, promoting proliferation of the AML cells. This would suggest that PP2A inhibition could be a recurrent event in AML. To test this hypothesis we first investigated the PP2A status in AML cell lines, observing an increased inhibition of PP2A in 11 out of 13 cell lines. Restoration of the PP2A phosphatase activity by treatment with forskolin in AML cells had antileukemic effects, blocking proliferation, and inducing caspase-dependent apoptosis. We also analyzed the status of previously described PP2A targets, and determined that enhanced PP2A activity induced by forskolin treatment decreased the levels of Akt and ERK1/2 phosphorylation, without affecting their expression. Moreover, there was an additive effect between forskolin and chemotherapic reagents idarrubicin and Ara-c, both used in standard induction therapy in AML patients. We next assessed whether PP2A was also altered in patient samples. Analysis at protein level of the PP2A activation status in 35 samples of patients with AML at diagnosis showed that all samples presented a reduced PP2A activity in comparison with normal donors. Altogether, these results suggest that functional inactivation of the PP2A tumor suppressor represents an important mechanism in AML transformation. To investigate other possible mechanisms that could be contributing to PP2A inhibition, we performed SNP and mRNA arrays in 12 AML cell lines. This approach allowed us to identify novel genomic aberrations affecting PPP2R5B and PPP2R5C expression. Our data suggest that loss of PPP2R5B and PPP2R5C, both PP2A subunits previously implicated in cancer, could be playing a role in AML development, contributing to deregulate the accurate PP2A function. Further studies are necessary to clarify the importance of these PP2A subunits in AML. In summary, we show that PP2A inhibition is a recurrent event in AML, and that restoration of the PP2A phosphatase activity in AML cells has antileukemic effects, showing an additive action in combination with idarrubicin and Ara-c. Therefore, PP2A activators, such as forskolin, could represent a future therapeutic alternative for treating patients with AML.

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AC220, A POTENT, SELECTIVE, SECOND GENERATION FLT3 RECEPTOR TYROSINE KINASE (RTK) INHIBITOR, IN A FIRST-IN-HUMAN PHASE 1 AML STUDY

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Background. Activating mutations in FLT3 RTK are present in ~30% of AML patients (pts), who have worse prognosis than wild-type (WT) pts. AC220 is a highly-selective 2nd generation RTK inhibitor of WT and mutant FLT3 with significant KIT activity. *Aims.* This was a Phase 1 open-label, dose escalation, first-in-man study to determine safety, tolerability, and PD of AC220 in AML pts. *Methods.* This study of predominantly relapsed/refractory AML pts was recently completed using a standard 3+3 dose escalation with 50% increments. Once-daily oral AC220 was administered initially with an intermittent dosing (ID) regimen: 14 on, 14 off (1 cycle). Starting dose was escalated from 12 to 450 mg/day. Additional continuous dosing (CD) cohorts were investigated: 200 and 300 mg/day for 28 days (1 cycle). *Results.* Seventy-six pts (46 male, 30 female) received AC220. Median age was 60 yrs (23-86 yrs), median prior therapies was 4 (0-8), with 16 pts having prior MDS or MDS/MPD, 11 pts having prior allogeneic transplant and 3 pts (≥72 yrs; unfit for induction chemotherapy) being previously untreated. Eighteen pts (24%) had FLT3-ITD mutations, 45 (59%) were ITD negative, and 13 (17%) undetermined. Pts were evaluated for PK and biomarkers such as phosphorylated (p) FLT3, pKIT, pSTAT5, and ex-vivo plasma inhibitory activity. AC220 plasma exposure was sustained between dose intervals and dose-proportionally increased from 12-450 mg with a half-life of ~1.5 days. Patient plasma at ≥12 mg potently inhibited pFLT3 in *ex-vivo* FLT3-ITD cell lines, with complete inhibition in WT cell lines observed at higher doses. Target inhibition was

observed, with suppression of pFLT3, pSTAT5 and pKIT in peripheral blasts. Most commonly reported possibly drug-related AEs were GI events, peripheral edema, and dysguesia, (predominantly Grade ≤2). DLT was observed at 300mg CD; 200mg CD was declared MTD. Two 300mg CD pts had possibly drug-related DLTs with Grade 3 QTc prolongation, but had confounding factors including concomitant QTc-prolonging medications. Responses (IWG criteria) were observed in 23 (30%) pts. PR and CR were observed as low as the 18 and 40mg cohorts, respectively. Most responses occurred within cycle 1. Overall, 10 (13%) pts had CR (2 CR, 6 CRi, and 2 CRp). One patient had complete resolution of leukemia cutis. Thirteen pts (18%) achieved PR. Median duration of response (MDOR) was 14 weeks (4-67+); median survival was 14 weeks (1-67+). In FLT3-ITD positive and negative pts, MDOR was 12 and 32 weeks. Ten (56%) of 18 FLT3-ITD pts were responders (1 CR, 4 CRi, 5 PR), 9/45 (20%) FLT3-ITD negative pts (1 CRi, 2 CRp, 6 PR), and 4/13 (31%) undetermined pts (1 CR, 1 CRi, 2 PR). At 200mg CD (MTD expansion), 4/6 FLT3-ITD positive pts responded (1 CR, 2 CRi, 1 PR). Of these responders, 2 failed prior sorafenib treatment and 2 refractory pts went onto transplant. The 2 non-responders had 6 and 8 prior lines of therapy, respectively. SUMMARY: These encouraging efficacy results and acceptable safety profile warrant continued evaluation of AC220 monotherapy and combination therapy in AML. Phase 2 studies in FLT3-ITD positive and negative pts are in progress.

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INDUCTION OF COMPLETE AND MOLECULAR REMISSIONS IN ACUTE MYELOID LEUKEMIA BY WILMS' TUMOR 1 ANTIGEN-TARGETED DENDRITIC CELL VACCINATION

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Background. Active immunization using tumor antigen-loaded dendritic cells (DC) holds promise for the adjuvant treatment of cancer to eradicate or control residual disease, but so far most DC trials have been performed in end-stage cancer patients with high tumor load. *Aims.* The majority of patients with acute myeloid leukemia (AML) will relapse and - except for allogeneic stem cell transplantation in subsets of patients - there is no consensus as to which post-remission treatment has to be applied in order to prevent relapse. Here, in a phase I/II trial, we investigated the effect of autologous DC vaccination in 20 patients with AML in remission but at high risk of full relapse. *Methods.* The Wilms' tumor 1 antigen (WT1), a nearly universal tumor antigen, was chosen as an immunotherapeutic target because of its established role in leukemogenesis and superior immunogenic characteristics. Myeloid DC were derived from immunoselected $CD14^+$ monocytes by culture with GM-CSF + IL-4, matured with TNF-α, prostaglandin E2 and in a majority of cases with keyhole limpet hemocyanin (KLH), electroporated with *WT1* mRNA and administered intradermally, at least 4 times on a biweekly basis. Quantitative levels of *WT1* mRNA in blood and marrow were followed as a marker of (minimal) residual disease. *Results.* Out of twenty patients, 17 are evaluable. Two out of 3 patients who were in partial remission with morphologically demonstrable disease after chemotherapy were brought into complete remission following intradermal administration of *WT1* mRNA-electroporated DC. In those 2 patients as well as in 7 other patients who were in complete remission but who had molecularly demonstrable residual disease, there was a return to normal of the *WT1* mRNA tumor marker following DC vaccination, compatible with the induction of molecular remission in 9/17 patients vaccinated thus far. Out of those 9 patients, 2 have relapsed with morphologically demonstrable disease and 4 have shown over the time signs of molecular relapse, as demonstrated by increase above normal background levels of *WT1* mRNA expression levels. In those 4 patients, *WT1* expression then usually normalised under further bi-monthly DC vaccination. Immunomonitoring, performed in 10 patients vaccinated with KLH-exposed DC vaccines, showed a significant increase in WT1-specific $CD8^+$ but not $CD4^+$ T cells and signs of general immune stimulation, such as a significant increase post-vaccination of plasma levels of interleukin 2 and of HLA-DR^+ $CD4^+$ T-cells. There was an inverse correlation between clinical response and degree of skin induration in delayed type hypersensitivity reactions to KLH alone. There was no significant change post-vaccination in WT1 antibody levels. *Conclusions.* Vaccination with *WT1* mRNA-loaded DC elicits immunological and clinical responses in AML patients. DC-based immunotherapy emerges as a feasible and effective strategy to control residual disease and prevent full relapse in AML.

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LONG-TERM OUTCOMES OF RESPONDERS IN A RANDOMIZED, CONTROLLED PHASE II TRIAL OF APTAMER AS1411 IN AML

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Background. AS1411 is the most advanced aptamer in oncology clinical development. It targets nucleolin, a protein upregulated on the surface of cancer cells. Data from a phase II study of AS1411 in relapsed and refractory AML were reported at ASCO 2009. In this update, we report follow-up data for the responders in the study. *Methods.* This randomized, multi-center phase II trial compared AS1411 plus high-dose cytarabine (HiDAC) to HiDAC alone as treatment for relapsed (≤3 previous lines of therapy) or refractory AML. Patients in cohort I were randomized 2:1 to receive AS1411 10 mg/kg/day IV CI days 1-7 + HiDAC 1.5 g/m^2 bid days 4-7 (AS1411-10), or HiDAC alone for 4 days (control). Following safety assessment, a second cohort was randomized to receive AS1411 40 mg/kg/day + HiDAC (AS1411-40) or HiDAC alone. Objectives were comparison of response rates (CR+CRp), safety and tolerability between groups. For this analysis, we collected data on post-remission therapy (PRT) and overall survival (OS) among responders. *Results.* 71 patients were randomized: 22 to AS1411-10, 26 to AS1411-40 and 23 to control. 67 patients were evaluable for safety (AS1411-10, 21; AS1411 40, 25; control, 21). Grade 3 and 4 toxicities were similar across all groups: febrile neutropenia, neutropenia, thrombocytopenia, and infections. Deaths within 28 days of treatment were: AS1411-10, 1/21; AS1411-40, 2/25; and control, 3/21. 59 patients were evaluable for response; AS1411-10, 21% (4CR/19); AS1411-40, 19% (2CR+2CRp/21); and control, 5% (1CRp/19). PRT and OS data for responding patients are tabulated. *Conclusions.* This phase II trial suggested that addition of AS1411 to cytarabine may enhance anti-leukemic activity and that the combination has an acceptable safety profile in patients with relapsed and refractory AML. Follow up suggests substantial survival durations in some patients responding to AS1411 + cytarabine. A phase IIb study is now evaluating responses, duration of responses and survival in AML patients randomized to AS1411 + cytarabine or cytarabine alone.

Table 1.

Group	CR/CRp	PRT	OS (months)
Control	CRp	None	6
AS1411-10	CR	Clinical trial	12
	CR	HiDAC	12+
	CR	HSCT	18+
	CR	HSCT	20+
AS1411-40	CRp	Allo SCT	3
	CR	DLIs, Depocyt	5
	CRp	-	14+
	CR	-	-

Granulocytes

1120

INHIBITION OF HISTONE DEACETYLASE ACTIVITY SUPPRESSES THE 60S SUBUNIT MATURATION DEFECT IN A YEAST MODEL OF SHWACHMAN DIAMOND SYNDROME

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Background. Shwachman Diamond Syndrome (SDS) is an autosomal recessive bone marrow failure syndrome that predisposes patients to developing leukemia. *SDO1* is the yeast ortholog of SBDS, the gene affected in SDS. Sdo1 is required for late stages of 60S subunit maturation. Strains depleted of Sdo1 contain half-mer polysomes as a consequence of the retention of pre-60S subunits in the nucleus. *Aims.* Recent studies have shown that certain classes of histone deacetylase inhibitors ameliorate the growth defect of *SDO1* mutants. Our goal was to identify mechanisms whereby histone deacetylase inhibitors rescued the growth defect of cells depleted of Sdo1. *Methods/Results.* We have found that trichostatin A showed a dose dependent increase in growth rate of cells depleted of Sdo1, with a corresponding resolution of half-mer polysomes. We have examined yeast cells extracts for proteins differentially acetylated between *SDO1* mutants and wild-type cells. Through the course of these studies we showed that a number of ribosomal proteins are subject to histone deacetylation and that a reciprocal relationship exists between the degree of acetylation of ribosomal proteins and the binding of Tif6 to 60S ribosomal subunits. Since the role of Sdo1 in 60S subunit maturation is thought to be mediated in part through its effect on the association of Tif6 with 60S subunits, these data suggest a mechanism whereby Sdo1 could influence subunit maturation by influencing protein acetylation. We also provide evidence that Sdo1 may influence translational accuracy through an effect on protein acetylation. *Summary/Conclusions.* These observations establish a previously unappreciated link between SBDS/Sdo1p, HDAC regulation, and 60S subunit maturation, and also uncover potential HDAC-directed therapeutic strategies for treating SDS and related diseases.

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ABNORMAL TELOMERE SHORTING IN PERIPHERAL BLOOD MONONUCLEAR CELLS AND GRANULOCYTES OF PATIENTS WITH CHRONIC IDIOPATHIC NEUTROPENIA

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Background. Abnormal telomere shortening in peripheral blood mononuclear cells (PBMCs) and/or granulocytes has been reported in patients with acquired bone marrow failure syndromes, including aplastic anaemia, myeolodysplasia syndromes, and paroxysmal nocturnal haemoglobinuria. The abnormality has been mainly attributed to the rapid turnover of haemopoietic progenitor cells in an attempt to compensate for the marrow failure. *Aims.* To evaluate the telomere length of PBMCs and granulocytes in patients with chronic idiopathic neutropenia (CIN). CIN is an acquired disorder of granulopoiesis characterised by increased apoptosis of the granulocytic progenitor cells and presence of activated T-lymphocytes in the peripheral blood and bone marrow. *Methods.* We studied 22 CIN patients (18 females, 4 males) aged 31 to 72 years (median 52.5 years) and 28 healthy individuals (23 females, 5 males) aged 31 to 74 years (median 51.5 years). DNA was extracted from peripheral blood granulocytes and PBMCs and telomere length was evaluated by means of real time PCR using β-globin as control single copy gene. Individual telomere length was reflected by the relative telomere/single-copy-gene (T/S) ratio based on the formula $T/S=2^{-\Delta Ct}$ ($\Delta Ct = Ct^{telomere} - Ct^{\beta\text{-globin}}$), in both cell populations tested. *Results.* Individual T/S ratio in the group of patients was characterized as appropriate or inappropriate for a given age by defining the observed/predicted (O/P) relative telomere length ratio for each cell population (granulocytes or PBMCs) on the basis of the equation derived from the linear regression analysis of the correlation between T/S ratio and age (years) of the controls. We found that the mean O/P telomere length ratio of patient PBMCs (0.43±0.21) was out of the lower 95% confidence limit of the healthy controls (mean O/P telomere length ratio 1.00±0.48; P<0.0001) suggesting inappropriate telomere loss by age in CIN patients. Regarding peripheral blood granulocytes, the mean O/P telomere length ratio in patients was within the 95% confidence limit of the controls. By analyzing, however, the mean T/S ratio per decade of years, we found that in the group of 31-40 years, CIN patients displayed significantly lower telomere length (3705±460, n=5) compared to controls (9481±5528, n=7) (P=0.01). Furthermore, the anticipated inverse correlation between granulocytes' T/S ratio and age was abrogated in the group of CIN patients but not in the healthy controls (r= -0.486, P=0.0087) suggesting that additional factors regulate granulocytes' telomere length in CIN patients. Finally, no association was found between absolute neutrophil counts and granulocyte T/S values in the group of CIN patients. *Summary/Conclusions.* Patients with CIN display inappropriate telomere loss in PBMCs compared to age- and sex-matched healthy individuals. This abnormality might be attributed to the increased activation of peripheral blood T-lymphocytes previously described in CIN. The granulocytes of CIN patients display an age-independent telomere length whereas inappropriate telomere loss may be seen in the younger age group. Overall, these data indicating inappropriate telomere loss in PBMCs and granulocytes of patients with CIN, support further the hypothesis that CIN shares common pathophysiologic features with other acquired bone marrow failure syndromes.

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MECHANISTIC INSIGHT INTO THE NAMPT / SIRT1 MEDIATED DOWN REGULATION OF P53 AND FOXO3A LEADING TO IMPAIRMENT OF GENES INVOLVED IN CELL CYCLE REGULATION AND DNA DAMAGE REPAIR

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Severe congenital neutropenia (CN) is a heterogeneous disorder of hematopoiesis characterized by a maturation arrest of granulopoiesis. CN is also considered as a pre-leukemic syndrome, since app. 20% of CN patients develop AML/MDS. Surprisingly no mutations in genes, which are typical for AML/MDS were detected in patients with CN who developed leukemia. But studies in CN patients reveal high association of G-CSF receptor mutation with the incidence of leukemia. In search of a factor dysregulated downstream of G-CSF receptor signaling we found that Nicotinamide Phosphoribosyltransferase (NAMPT), a protein involved in biosynthesis of NAD+ was significantly increased in CN patients treated with G-CSF as compared to healthy individuals (Skokowa *et al.*, Nature Medicine, 2009). Increased NAD^+ levels correlated with the elevated levels of SIRT1, a NAD^+-dependent deacetylase involved in the deacetylation of histone and non-histone proteins. The tumor suppressor p53 and FOXO3a are among the non-histone proteins targeted by SIRT1 therefore we asked if deacetylation dependent inactivation of p53 and FOXO3a plays a role in the leukemic transformation in CN. In this study we demonstrate that presence of NAMPT or NAD^+ enhances the activity of SIRT1 to deacetylate p53 and FOXO3a. The compound FK866 specifically inhibits NAMPT and has recently entered clinical trials as a potential chemotherapeutic agent. We show that the treatment of promyelocytic leukemia cell line NB4 with FK866 increases the endogenous acetylation of p53 and FOXO3a proteins and this increased acetylation is in part due to decreased interaction of p53 or FOXO3a protein with SIRT1. The cell cycle regulator p21 and DNA damage repair protein GADD45A were among the target genes down regulated in $CD34^+$ and NB4 cells on treatment with NAMPT and the levels of these both genes were rescued on use of FK866. In reporter gene assay we show that the presence of NAMPT or NAD^+ increases the ability of SIRT1 to inhibit the p53 and FOXO3a mediated activation of p21 and GADD45A promoter respectively. Additionally use of FK866 rescues the ability of p53 and FOXO3a to activate p21 and GADD45A promoter activity by inhibiting NAMPT/SIRT1 pathway. Knockdown of p53 or FOXO3a using specific shRNA against these proteins inhibits the expression of p21 or GADD45A respectively. Further inhibition of NAMPT/SIRT1 pathway using shRNA against SIRT1 leads to increased expression of p21 and GADD45A mRNA. Taken together our working hypothesis is that NAMPT/NAD^+ activated SIRT1 mediates deacetylation of p53 and FOXO3a leading to downregulation of downstream target genes p21 and GADD45A respectively. This inhibition of tumor suppressor functions of p53 and FOXO3a might possibly be involved in the leukemic transformation in CN.

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THE RISK OF LEUKEMIA IN GENETIC SUBGROUPS OF CONGENITAL NEUTROPENIA

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An increased risk for malignant transformation (MDS or leukemia) is well documented in patients with congenital neutropenia (CN). In this study we assessed the incidence of leukemic transformation and potential risk factors for leukemic transformation in CN patients with known gene mutations, e.g. ELA2, HAX1, G6PC3, p14, WAS or no identified mutation, respectively, by combining all available data from the European and US Branches of the Severe Chronic Neutropenia Registry (SCNIR). Data from mutational analysis were available for 369 patients. Mutations were identified in 206 CN patients, of whom 149 patients revealed ELANE mutations, 23 HAX1 mutations, 20 WASP, 10 G6PC3 and 4 p14 mutations. In addition, in 35 patients neither ELANE nor HAX 1 mutation were detectable and in another 41 patients ELANE mutations could be excluded, but further genetic evaluation is not yet completed. Mutational analysis were also available in 87 patients with cyclic neutropenia, of whom 63 revealed ELANE mutations and 24 were negative for ELANE mutations. Secondary malignancies occurred in 45 of the 281 CN patients. The distribution by genetic subtype is shown in the Table below:

Table. Congenital Neutropenia subtype by gene mutation Total patient number (n) MDS/Leukemia (n/%).

ELANE-CN	149	27 (18.1%)
HAX1-CN	23	4 (17.4%)
ELANE neg + HAX1 neg	35	6 (17.1%)
ELANE neg/ HAX1 nt	41	4 (9.8%)
WAS	20	4 (20%)
G6PC	3	10 0
p	14	4 0
ELANE+Cyclic Neutropenia	63	1 (0.01%)
ELANE- Cyclic Neutropenia	24	0

All subgroups benefit from G-CSF treatment. Median G-CSF maintenance doses required during the years prior to leukemic transformation differ significantly between patients with or without leukemia. *Conclusion.* Patients with severe congenital neutropenia who have mutations in ELANE, HAX1, or WAS as well as those with no recognized mutation are at risk of secondary leukemia. So far, progression to MDS leukemia has not yet been described in the small number G6PC3 or p14 CN cases in our database. Patients requiring higher doses of G-CSF are at greater risk. Despite mutations in the ELANE gene patients with cyclic neutropenia exhibit no increased risk for malignant transformation. Mutational analysis is helpful to identify the genetic cause of severe congenital or cyclic neutropenia but does not serve to identify patients at risk of leukemic transformation.

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INHIBITION OF THE NAMPT/SIRT2 PATHWAY LEADS TO THE REDUCED PROLIFERATION AND INCREASE APOPTOSIS OF THE ACUTE LEUKEMIA CELLS VIA MODULATION OF THE AKT/GSK3β PATHWAY

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Recently we demonstrated that nicotinamide phosphoribosyltransferase (NAMPT) is an essential enzyme mediating granulocyte colony-stimulating factor (G-CSF)-triggered granulopoiesis and that sirtuin-1 activation is a key molecular event during this process. Sirtuins are the members of the NAD^+-dependent class III histone deacetylases, and they have been associated with many physiological and patholo gical processes. We herein investigated whether NAMPT is involved in leukemogenesis by mediating SIRT dependent deacetylation. Screening of the mRNA and protein levels of the different sirtuins in primary acute myeloid leukemia blasts revealed significant upregulation of SIRT2 mRNA and protein. Therefore, we evaluated the role of SIRT2 in leukemogenesis and potential mechanisms of SIRT2 activation via NAMPT. We found that both specific inhibition of NAMPT (using 10 nMol of FK866) or SIRT2 (using 100nMol of AC93253) significantly reduced proliferation and induced apoptosis in a wide range of human myeloid leukemia cell lines (NB4, HL60 and U937). NAMPT inhibition resulted in the reduced activity of SIRT2, as assessed by the colorimetric SIRT2 activity assay. Interestingly, we additionally observed that treatment with AC93253 (25nM) did not affected the proliferation of $CD34^+$ bone marrow progenitor cells of healthy individuals, but inhibited their G-CSF-triggered myeloid differentiation. This suggested that different mechanisms are operating downstream of NAMPT in the "normal"and leukemogenic myeloid cells. The anti-tumour activity of FK866 and AC93253 was accompanied by hyperacetylation of α-tubulin, inhibition of the activation of Akt via phosphorylation on Thr308 and Ser473 and elevated activation of GSK-3β via inhibition of phosphorylation on Ser9. GSK-3β is a known inhibitor of Wnt signaling pathway and we demonstrated that treatment with FK866 was accompanied by diminished expression of Wnt target genes LEF-1 and cyclin D1. Taken together, our results provide strong evidence that NAMPT and SIRT2 participate in leukemogenesis and that Akt/GSK-3β pathway acts as a target in FK866- or AC93253-induced inhibition of the proliferation of leukemia cells. This is the first report on the role of Sirt2 in leukemias.

Clinical stem cell transplantation

1125

HIGH DOSE THERAPY AND AUTOLOGOUS STEM CELL TRANSPLANTATION IN FIRST RELAPSE FOR DLBCL STILL IMPROVES PROGRESSION FREE SURVIVAL IN THE RITUXIMAB ERA. A RETROSPECTIVE ANALYSIS OF THE EBMT-LWP

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Background. Autologous stem cell transplantation (ASCT) remains the treatment of choice for patients (pts) with diffuse large B cell lymphoma (DLBCL) that relapse after first line chemotherapy (CT). Nevertheless, the impact of the use of the anti-CD20 monoclonal antibody (Rituximab) (RTX) with combination CT on the ulterior results of the transplantation procedure has to be determined. *Aims.* This study was designed to evaluate the benefit of this strategy, in pts with DLBCL achieving after salvage CT a 2nd complete remission (CR2), by retrospectively comparing for each pt the progression free survival (PFS) after ASCT with the duration of the previous CR. *Methods.* Adult DLBCL pts with MEDB information available autografted in CR2 between 1990 and 2005 in EBMT centres were included in the analysis. A total of 470 pts (262 males, median age 52 (18-74) years] were evaluated. 351 pts (74%) did not receive RTX prior to ASCT, 119 pts (25%) did receive it. Duration of CR1 was 11 (1-112) months [median (range)]; it lasted less than 12 months in 49% of the cases. Median time from diagnosis to ASCT was 24 (6-395) months. Peripheral blood was used as the source of hematopoietic stem cells in 399 pts (85%). The BEAM protocol was the conditioning regimen most frequently used (n=258, 63%) and only 5.5% pts were conditioned with TBI-containing regimens. *Results.* After a median follow up after ASCT for surviving pts of 52 months, 5 years overall survival (OS) was 63% and PFS 48%. 196 (41%) pts did relapse after ASCT [median (range), 10 (1-172) months] and 35 (7%) pts died from non-relapse mortality. Factors negatively affecting PFS were treatment before 2002 (P=0.01) and age over 50 years (P=0.004). Patients treated after 2002 with RTX at first line treatment or at relapse had a better PFS (80%) than patients treated with RTX at first line and at relapse (56%). In 289 patients the PFS period after ASCT was longer than the previous CR1 duration and in 121 cases the PFS period was shorter. This difference in favour of post ASCT period was significant for patients with or without prior exposure to RTX (P<0.01) and age >50 years or <50 years (P<0.001). Finally, when each patient was taken as his/her own control, PFS after ASCT was longer than PFS for CR1 (P<0.001). *Conclusions.* The use of RTX prior to ASCT did not impair the beneficial effects of the autologous procedure in the whole population of pts (RTX yes: 58% *vs.* no 41%, (P<0.05)). 2-years PFS after ASCT was significantly lower in pts with a CR1 < 12 months (P<0.005). ASCT can significantly increase PFS in comparison with the duration of CR1 in DLBCL and can modify disease course. The use of RTX prior to ASCT does not decrease the beneficial effect of pts autografted in CR2 when compared to their prior CR1 duration. The duration of CR1 remains one of the most important prognostic factors for ASCT outcome.

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CONSTITUTIONAL VARIABILITY IN GENES INVOLVED IN INNATE IMMUNITY AND IN CELL PROLIFERATION INFLUENCES DISEASE FREE SURVIVAL AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

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Background. Innate immunity plays an important role in infections and in activation of adaptive immunity modulating the intensity of the inflammatory response after allogeneic stem cell transplantation (allo-SCT). Thus, genetic variability in donor and recipient in these genes and in genes involved in cell proliferation might be an important factor influencing the outcome of allo-SCT. *Aims.* To study the potential influence of 15 single nucleotide polymorphisms (SNPs) in donor and recipient in genes of innate immunity (IRF-3, HAMP, PTX3, HBD2) and regulation of cell proliferation (ATBF1, NFAT5, AKT2, NM1, CD151, TCIRG1, SH3KBP1) on clinical outcomes after allo-SCT, specifically on the incidence of acute GvHD (aGvHD), chronic GvHD (cGvHD), presence of infections, transplant related mortality (TRM), relapse, and disease free survival (DFS). *Methods.* Study population consisted of 106 donor-patient pairs undergoing HLA identical sibling allo-SCT in a single institution. Patient median age was 38 years (range, 5-66). 42% of the patients were in advanced phase of disease. Cumulative incidence for GvHD, TRM, relapse and DFS was computed with the cmprsk package and with Kaplan-Meier. Analysis of GvHD and infections were restricted to 87 donor-patient pairs. *Results.* Patient IRF3 rs2304205 AA, donor ATBF1 rs719327 AA and patient AKT2 rs12460555 CC dominant genotypes, were associated with a higher incidence of relapse (P=0.02, P=0.04 and P=0.002, respectively) and lower DFS (P=0.02, P=0.04 and P=0.009, respectively). All of them retained significance at multivariate analysis. Variant rs719327 AA in ATBF1 showed the same prognostic values when present in donor or in patient (relapse: 55% *vs.* 34% and DFS: 26% *vs.* 46%). When it was present both in donor and patient, the differences were more prominent (relapse: 69% *vs.* 33%, P=0.003 and DFS: 15% *vs.* 45%, P=0.01). Donor HAMP rs7251342 GG recessive genotype and donor NFAT5 rs6499244 AA dominant genotype were associated with a higher incidence of TRM (P=0.016 and P=0.02, respectively) influencing in lower OS and DFS (P=0.0016 and P=0.02, respectively). They retained its significance at multivariate analysis. Genetic variant in donor for HAMP and in patient for NFAT5 were associated with a higher and lower frequency of infections (P=0.04 and P=0.05, respectively). NFAT5 is necessary for optimal T cell development and rs6499244 AA variant showed the highest mRNA expression. Donor IRF3 rs7251 CC and patient EP300 rs20551 AA genotypes were associated with a higher incidence of cGvHD (P=0.023 and P=0.049, respectively) influencing in a lower DFS (P=0.012 and P=0.009, respectively) and OS (P=0.02 and P=0.02, respectively). None of the 25 patients with a donor carrying PTX3 rs18040680 AA recessive genotype had TRM but patients carrying this genotype showed higher frequency of infections (P=0.04). *Conclusions.* Genetic variability in innate immunity and in cell proliferation has a strong influence on the clinical outcome of allo-SCT, which might be important when choosing allo-SCT protocols.

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A NOVEL HIGH DOSE CHEMOTHERAPY STRATEGY WITH BENDAMUSTINE IN ADJUNCT TO ETOPOSIDE, ARACYTIN AND MELPHALAN (BEEAM) FOLLOWED BY AUTOLOGOUS STEM CELL RESCUE IS SAFE AND HIGHLY EFFECTIVE FOR THE TREATMENT OF RESISTANT/RELAPSED LYMPHOMA PATIENTS: A PHASE I-II STUDY ON 33 PATIENTS

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Background. Carmustine, etoposide, cytarabine, and melphalan (BEAM) regimen is the most used conditioning regimen to proceed to autologous stem cell transplant (ASCT) in lymphoma patients. However, those patients receiving BEAM show a significant number of side effects, and relapse rate after transplant is still a matter of concern. Therefore, new regimens with a higher efficacy and a better toxicity profile in comparison to BEAM are highly needed. *Aims.* We designed a phase I-II study to evaluate the safety and the efficacy of increasing doses of Bendamustine for the conditioning regimen to ASCT for resistant/relapsed lymphoma patients. *Methods.* Thirty-three patients (median age 51 years, range 18-70) with resistant/relapsed non-Hodgkin (23) or Hodgkin (10) lymphoma were consecutively enrolled in the study.

The new conditioning regimen consisted of increasing doses of Bendamustine coupled with fixed doses of Etoposide (200mg/m^2/day on days -5 to -2), Cytarabine (400mg/m^2 on days -5 to -2) and Melphalan (140 mg/m^2 on day -1) (BeEAM regimen). Three cohorts of 3 patients each were treated starting with Bendamustine 160 mg/m^2/daily given on days -7 and -6. The dose of Bendamustine was then escalated according to the Fibonacci's increment rule until the onset of severe adverse events and/or the attainment of the expected MTD, but not higher than 200 mg/m^2. Patients were carefully monitored for adverse events. The study was registered at EMEA with the EUDRACT no 2008-002736-15. *Results.* The administration of Bendamustine was safe in all the 3 cohorts of patients. The major side effect was a grade III-IV oral mucositis developed by 4 patients during neutropenia. We then fixed the dose of Bendamustine 200 mg/m^2 as safe and effective for the Phase II study. A median number of 6.1×10^{6}CD34$^+$/kg cells (range 2.4-15.5) collected from peripheral blood was reinfused to patients. All patients engrafted, with a median time to ANC>0.5×10^9/L of 10 days. Median times to achieve a platelet count >20×10^9/L and >50×10^9/L were 12 and 15 days respectively. Sixteen out of 33 patients presented a fever of unknown origin (48%). The median number of days with fever was 2 (range: 0-7), with a median number of 9 days of intravenous antibiotics (range: 3-22). All patients received G-CSF after transplant for a median time of 8 days (range: 8-13). Twenty-two out of 33 patients are evaluable up to now for the response to treatment. All evaluable patients are alive. 20/22 are in complete remission whereas 2/22 are in partial response, after a median follow-up of 7 months from transplant. It is of note that 3/22 patients achieved the first complete remission after receiving the high-dose therapy with autologous stem cell rescue. *Conclusions.* The new BeEAM regimen is safe and seems to have a high efficacy in heavily pretreated lymphoma patients. All the future studies who want to incorporate Bendamustine on such conditioning regimens for ASCT in lymphoma patients have to use 200 mg/m^2/day of Bendamustine given overt 2 days.

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ACUTE LYMPHOBLASTIC LEUKAEMIA IN CHILDHOOD: OUTCOMES OF UNRELATED CORD BLOOD TRANSPLANT

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Allogeneic stem cell transplantation (SCT) is indicated in approximately 20-30% of childhood acute lymphoblastic leukaemia (ALL). Unrelated umbilical cord blood (UCB) is an established stem cell source for SCT. We retrospectively analyzed 532 children with ALL in complete remission (CR)1 (n=186), CR2 (n=238) and CR3 or advanced disease (n=108) who received UCBT as first transplant. Patients were transplanted in EBMT centres from 2000-2008. Median age at transplant was 6.8 years (y) (29 patients less than 1y). The most common immunophenotype was B-cell precursor ALL and 17 patients had biphenotypic ALL. Of 186 patients transplanted in CR1, 45% had poor risk cytogenetics (t4;11 or t9;22).Grafts were composed of one (n=504) or two (n=28) units; 62% had 0-1 HLA mismatch with recipients while 38% had 2-3 mismatches (antigen level for HLA-A and B, allelic level for DRB1). Median TNC cell dose at freezing and infusion were 5.9×10^7/kg and 4×10^7/kg, respectively. Myeloablative conditioning regimen and TBI>6 Gy were used in 96% and 52% of cases, respectively. Other regimens included busulphan with cyclophosphamide ±thiotepa or melphalan. ATG was used in 88% of cases. GVHD prophylaxis was CSA ±steroids in 75%. Median follow-up was 18.5 months (3-109). Cumulative incidence (CI) of neutrophil recovery (NR), acute GVHD and TRM were 82±2%, 27±3% and 21±3%, respectively. In multivariate analysis, TNC infused >4×10^7/kg (P=0.001) and remission status at UCBT (P=0.01) were associated with improved NR. CI of 2y relapse was 37±3% (31% for CR1, 34% for CR2, 50% for advanced). Disease status at UCBT (HR=0.36, P= 0.001) and use of TBI>6Gy (HR=0.58, P=0.01) were independently associated with lower CI of relapse. The 2y probability of leukemia-free-survival (LFS) was 38±2% (49% for CR1, 42% for CR2, 10% for advanced; P=0.001). In multivariate analysis, disease status at UCBT was the only factor associated with improved LFS (HR=0.32, P=0.001). Causes of death were infections or other transplant-related events (n=172) or disease progression (n=95). In conclusion, in the absence of an HLA identical donor, UCBT remains a valuable alternative option for children with high risk ALL. Disease status at transplantation and cell dose are the most important factors influencing transplant outcome. Use of TBI in the conditioning regimen decreases the incidence of relapse but is not associated with improved LFS. The role of minimal residual disease as a predictor of outcomes of UCBT is currently under investigation.

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LATE PROPHYLACTIC DONOR LYMPHOCYTE INFUSIONS IN HIGH RISK ACUTE LEUKEMIAS

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Background. Prophylactic donor lymphocyte infusions (pDLI) given early (i.e. before day +100) after allogeneic hematopoietic stem cell transplantation (HSCT) have been shown to convert mixed to full donor chimerism in T cell-depleted stem cell transplants and to enhance the graft-versus-leukemia effect, however, at the expense of excessive graft-versus-host disease (GvHD). We prospectively studied pDLI administered late (i.e. after day +120) in high risk acute leukemias (AL). PATIENTS AND *Methods.* From 2004 to 2009, 279 patients received a T-cell replete allogeneic HSCT at our institution for AL at high risk of relapse. High risk criteria included adverse cytogenetics, relapsed or refractory disease, extramedullary leukemia, MDS with excess blasts, and therapy-related AML. 114 patients had *de novo* acute myelogenous leukemia (AML), 118 advanced myelodysplasia or secondary AML (MDS/sAML), and 47 acute lymphoblastic leukemia (ALL). 126 were females and 153 males, with a median age of 52 years (range: 17 to 71). Among these, 43 patients (14 AML, 25 MDS/sAML, 4 ALL) who did not have experienced GvHD even after withdrawal of immunosuppression, were treated with pDLI between days +125 and +469. Unmodified pDLI were started at 5×10^{5}CD3$^+$/kg and increased monthly in a maximum of two steps up to 1×10^7 CD3$^+$/kg, unless symptoms of GvHD occurred. pDLI were not given to the other 236 high risk AL patients due to prior GvHD, early relapse, recurrent infectious complications, or patient refusal. *Results.* 23 patients received all three doses of pDLI, 15 patients two, and 5 patients only the first dose. 9 of the 43 patients developped acute GvHD grade II to IV or extensive chronic GvHD after pDLI (five-year GvHD incidence 23%), compared to 51% in survivors beyond day +120 of the high risk AL patients not given pDLI (P=0.001), and compared to 58% in survivors beyond day +120 of high risk AL patients transplanted during the previous six years, 1998 to 2003, at our institution (P<0.001). Leukemic relapse occurred in 9 of the 43 patients despite pDLI (five-year relapse incidence 28%), compared to 45% in day-120-survivors of high risk AL patients without pDLI (P=0.020), and compared to 52% in day-120-survivors of historical controls (P=0.008). With 29 months of median follow-up (range: 5 to 61), five-year overall (OS) and disease-free survival (DFS) probabilities of pDLI patients are 76% and 71%, respectively, compared to 47% (P=0.001) and 42% (P=0.001) of day-120-survivors of the contemporary high risk AL patients, and 30% (P<0.001) and 25% (P<0.001) of the historical controls. When pDLI patients are compared with all contemporary and historical controls, respectively, differences in relapse rate, GvHD incidence, OS and DFS remain highly significant (P<0.001). *Conclusions.* In patients without GvHD after T-cell replete HSCT for high risk acute leukemias, unmodified prophylactic DLI after day +120 are safe and effective, reducing the relapse rate as well as the incidence of severe GvHD, and improving both overall and disease-free survival.

Novel rx approaches

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CLINICAL ACTIVITY IN A PHASE 1 STUDY OF CAL-101, AN ISOFORM-SELECTIVE INHIBITOR OF PHOSPHATIDYLINOSITOL 3-KINASE P110DELTA, IN PATIENTS WITH B-CELL MALIGNANCIES

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Background. The class I phosphatidylinositol 3-kinases (PI3Ks) regulate a variety of cellular functions relevant to oncogenesis. Expression of the PI3K p110δ isoform (PI3K_) is restricted to cells of hematopoietic origin where it plays a key role in B cell proliferation and survival. In chronic lymphocytic leukemia (CLL) and non-Hodgkin lymphoma (NHL) cells, constitutive PI3Kδ-dependent PI3K pathway activation is frequently observed. CAL-101 is an isoform-selective inhibitor of PI3Kδ (EC_{50} of 8 nM in a cell-based assay with >200-fold selectivity relative to other PI3Ks) that inhibits PI3K signaling and induces apoptosis of malignant B cells *in vitro*. *Aims.* A Phase 1 study was undertaken to evaluate the safety and activity of CAL-101 in patients with relapsed or refractory hematologic malignancies. *Methods.* Sequential dose escalation cohorts of 3+3 patients were enrolled to determine dose limiting toxicity (DLT). Subsequently, cohorts of patients with CLL, indolent NHL, aggressive NHL, acute myeloid leukemia (AML) and multiple myeloma (MM) were enrolled. CAL-101 was administered orally twice daily (BID) continuously for 28 day cycles for up to 12 cycles. Clinical response was evaluated according to standard criteria. Written informed consent was obtained from all patients. *Results.* At data cutoff, 90 patients were enrolled: 29 patients with CLL, 19 with indolent NHL, 18 with aggressive NHL, 12 with AML and 12 with MM. Patient characteristics were 36% female, median age 66, 51% had refractory disease and median number of prior therapies was 5. Dose levels were 50 mg (n=3), 100 mg (n=3), 150 mg (n=32), 200 mg (n=35) and 350 mg (n=17) BID. The median duration of treatment was 3 cycles, with 31% completing ≥6 cycles. Three patients completed the study with 48 weeks of therapy and 29 remain on study. Eight patients discontinued for adverse events, most frequently due to the DLT of increased ALT/AST. The DLT appears to be dose dependent with an incidence of 24% at 350 mg, 20% at 200 mg and 9% at 150 mg. ALT/AST abnormalities were reversible and most affected patients were able to resume at a lower dose. Hematological toxicity was minimal. 83 patients were evaluable for clinical response and 25 patients had objective responses, all partial responses. The response rate was 65% (11/17) in indolent NHL patients, 75% (6/8) in mantle cell lymphoma and 30% (8/27) in CLL. None of the 31 patients with diffuse large B-cell lymphoma, AML or MM had a response. Clinical responses were observed at all dose levels. Of 27 CLL patients, 93% had >50% reduction in lymphadenopathy which was commonly associated with a transient increase in peripheral blood lymphocytosis. Plasma exposure increased with dose in a less than dose-proportional manner. PI3K signaling was evaluated in 7 CLL patients and expression of phospho-AKT (T308) in circulating leukemic cells was found to be reduced by >90% following CAL-101 dosing. Updated data will be presented. *Conclusions.* CAL-101, an oral PI3Kδ isoform-selective inhibitor, shows promising clinical activity with acceptable toxicity in patients with indolent B-cell malignancies and mantle cell lymphoma.

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A PHASE I/II STUDY OF IPH1101, GAMMA DELTA T CELL AGONIST, IN COMBINATION WITH RITUXIMAB RE-TREATMENT, IN PATIENTS WITH LOW GRADE FOLLICULAR LYMPHOMA

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Background. Non-conventional γδ T lymphocytes have potent antitumoral activity, particularly against malignant B cells. IPH1101 is an agonist of γδ T cells, which in the presence of low doses of IL-2 potentiates their direct cytotoxic activity. ADCC is a major molecular mechanism underlying rituximab's efficacy. Increasing the number and the activation state of killer lymphocytes mediating ADCC is therefore believed to be beneficial for therapeutic potency. Since γδ T cells have been found to be capable of mediating ADCC, modulating γδ T cells in the context of rituximab is worth being tested in a clinical trial. *Aims.* The main purpose of this study was to assess the clinical efficacy of IPH1101 associated with low doses of IL-2, and combined with a standard rituximab treatment, in patients (pts) with follicular lymphoma (FL). *Methods.* This was an open label, multinational study consisting of a dose escalation Phase I like part followed by a phase II part. The phase I part showed a good safety and immuno-biological efficacy profile for the highest dose of IL-2. Consequently, the pts of the phase II part were treated with the combination of rituximab (375 mg/m^2) administered 4 times weekly, IPH1101 (750 mg/m^2) administered i.v. 3 times (every 3 weeks) and IL-2 (8 MIU) administered daily s.c. for 5 days starting on the day of each IPH1101 administration. All pts presented low grade FL which had relapsed after 1 to 4 lines of previous therapy including at least one rituximab-containing line. Inclusion criteria set forth that pts should have no lesion > 7 cm. The primary end point of the trial was the rate of overall response at 6 months. All patients enrolled in the trial had signed informed consent. *Results.* Interim data analysed on the first set of patients (41 for safety and 34 patients assessed for efficacy after independent central review showed a good overall safety, with most of the drug-related adverse events being flu like symptoms of grade 1 or 2. The SAEs reported were 2 hypotensions, 1 bronchospasm, 1 allergic reaction (back pain), 1 glomerular filtration decrease, 1 ALAT elevation, 1 diarrhea, 1 pyrexia, and 1 asthenia. The immuno-biological follow up demonstrated the very good pharmacodynamic profile of IPH1101 in these patients. The overall response rate for this cohort of 34 evaluable patients (20 patients at 6 months, and 14 at 3 months), showed an Objective Response Rate of 47%, and an encouraging 32% Complete Response rate. *Summary/Conclusion.* The response rate in this first set of pts is encouraging in the context of previously treated patients. To date the enrolment has been completed with 45 patients treated, and the 6 month follow up for the last patient has been reached. Independent review is currently on going for the last set of patients, and complete efficacy study results will be presented at the meeting.

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PBI-1402: A FIRST-IN-CLASS ERYTHROPOIESIS-STIMULATING AGENT (ESA) WHICH REDUCES THE NEED FOR BLOOD TRANSFUSION IN CHEMOTHERAPY-INDUCED (CIA) ANEMIC PATIENTS

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Background. PBI-1402 is a first-in-class novel orally active compound that promotes the production of erythrocytes by a mechanism of action distinct from erythropoietin (EPO). This mechanism involves differentiation of earlier progenitor stem cells (CFU-GEMM) than those stimulated by EPO (BFU-E, CFU-E). In a phase I clinical trial, PBI-1402 induced a significant increase (100%, P<0.001, compared to placebo) of relative and absolute reticulocyte count in healthy volunteers after 21

days of oral treatment and was devoid of significant side effects. Furthermore, a phase Ib/II clinical trial confirmed that PBI-1402 increased red blood cell (RBC) counts and hemoglobin (Hb) levels in patients with chemotherapy-induced anemia (CIA). *Aims.* The objectives of this extension clinical phase Ib/II trial were to provide additional data on the safety and tolerability of PBI-1402 and to assess its biological efficacy (including the reduction in the need for transfusion) in patients with CIA. *Methods.* PBI-1402 was administered orally once a day for eight weeks to patients undergoing chemotherapy for various cancers. Patients were assessed every two weeks for safety, tolerability, Hb level, RBC count and clinical biochemistry. The results presented are a combination of the clinical phase Ib/II trial and its extension study. The former consisted of a dose-ranging study of three cohorts of six patients receiving 44, 66 and 88 mg/kg. The latter consisted of 12 patients receiving 44 mg/kg. *Results.* Of the 30 patients enrolled, 29 patients completed their eight-week PBI-1402 treatment. PBI-1402 was well tolerated and no severe side effects were observed. Blood chemistry remained within the normal range of clinical values. 93% of patients (27/29) did not require blood transfusion since their RBCs and Hb increased or remained stable. Moreover, of the 50% of patients who experienced a significant increase ($P<0.001$ relative to their baseline) in their RBCs and Hb, none reached levels currently proscribed by the most recent therapeutic guidelines (no overshoot). No change in white blood cell and platelet counts were observed in this study. *Conclusion.* PBI-1402 is safe and well tolerated. 50% of the patients displayed a significant increase in RBC count and Hb level. Of the 29 patients that completed the study, 93% did not require blood transfusion. Furthermore, preclinical evidence of antitumor activity suggests that PBI-1402 is safe to use in the management of anemia associated with cancer or induced by chemotherapy. Therefore, orally administered PBI-1402 offers significant potential for the treatment of anemia associated with cancer and/or chemotherapy, an unmet medical need.

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ANTITUMOR ACTIVITY OF THE INVESTIGATIONAL DRUG MLN9708, A SECOND-GENERATION PROTEASOME INHIBITOR, IN PRECLINICAL MODELS OF DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL)

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Background. The ubiquitin-proteasome system is involved in the degradation of most cellular proteins. The successful development of bortezomib for multiple myeloma and previously treated mantle cell lymphoma has validated the proteasome as a therapeutic target for hematological malignancies. The investigational drug MLN9708, like bortezomib, is a modified dipeptidyl boronic acid that is a potent, reversible and specific inhibitor of the 20S proteasome. MLN9708 is currently in Phase I trials for the treatment of hematologic malignancies as well as solid tumors. *Methods.* We evaluated the ability of MLN9708 to inhibit tumor growth in four preclinical models of diffuse large B-cell lymphoma (DLBCL) which represent several genetic subgroups of this disease including activated B-cell-like (ABC-DLBCL), germinal-center B-cell-like (GCB-DLBCL), and a third group that is distinct from both subgroups (non-ABC and non-GCB-type DLBCL). We aimed to determine whether MLN9708 has broad activity in preclinical models of DLBCL, or whether antitumor activity is subtype-specific. MLN9708 immediately hydrolyzes to MLN2238, the biologically active form, upon exposure to aqueous solutions or plasma. MLN2238 was used for all preclinical studies described below. Immunocompromised mice were implanted with subcutaneous xenografts of OCI-LY10 (ABC-DLBCL), PHTX-22L (ABC-DLBCL), and WSU-DLCL2 (non-ABC and non-GCB-type DLBCL) and dosed once or twice-weekly for three weeks. The treatment over control (T/C) ratio of the mean tumor volume on the last day of the study was used as a measure of antitumor activity. To generate a disseminated model of lymphoma, OCI-LY7 (GCB-DLBCL) cells were luciferase-tagged (OCI-LY7-luc) and inoculated IV into immunocompromised mice, and tumor burden was monitored over time via bioluminescent imaging. *Results.* MLN2238 showed antitumor activity in models classified as ABC-DLBCL. For the OCI-Ly10 xenograft model, once-weekly (QW) IV dosing of MLN2238 at 18 mg/kg (MTD) resulted in T/C of 0.12 and induced tumor regression in 6 of 7 mice, while doses of 8 mg/kg and 4 mg/kg IV QW resulted in T/C of 0.34 and 0.36 in separate studies. PHTX-22L is a primary human tumor xenograft derived from a surgically resected lymph node from a patient diagnosed with DLBCL. MLN2238 showed significant antitumor activity against PHTX-22L at 14 mg/kg twice weekly (BIW), 11 mg/kg BIW, 7 mg/kg BIW, and 11 mg/kg QW ($T/C \leq 0.15$). However, 7 mg/kg QW or 3.5 mg/kg BIW in this model was less efficacious ($T/C=0.76$), consistent with a dose-dependent level of tumor proteasome inhibition. MLN2238 also showed antitumor activity in models that are not classified as ABC-DLBCL. In the WSU-DLCL2 model (non-ABC and non-GCB DLBCL), MLN2238 at 14 mg/kg BIW showed $T/C=0.44$, and this dose provided sustained tumor proteasome inhibition and activated downstream consequences including apoptosis. In the OCI-LY7-luc disseminated model of GCB-DLBCL, MLN2238 reduced tumor burden and improved overall survival compared to vehicle treated controls (median survival of 54 *vs.* 33 days). *Conclusions.* These studies demonstrate that MLN2238 is active in preclinical models of DLBCL across multiple subtypes. Strikingly, all four of the DLBCL models showed minimal or no tumor growth inhibition in response to bortezomib, highlighting the improved antitumor activity of MLN2238 in preclinical models.

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LEUKEMIC CD52 NEGATIVE GPI DEFECTIVE CLONES ARE COMMON IN ALL, ESCAPE ALEMTUZUMAB THERAPY, BUT ARE SENSITIVE TO RITUXIMAB MEDIATED COMPLEMENT DEPENDENT CYTOTOXICITY: RATIONALE FOR COMBINATION THERAPY

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B-lineage acute lymphoblastic leukemia (B-ALL) in adults is sensitive to polychemotherapy but relapses frequently occur. B-ALL frequently express CD52, and since Alemtuzumab (ALM) is directed against the glycophosphatidyl-innositol (GPI-) anchored membrane protein CD52, ALM may be of therapeutic value. However, loss of CD52 due to acquired defects in GPI biosynthesis has been observed in various hematological disorders. GPI-defective leukemic subclones in ALL will result in resistance to ALM. We analyzed CD52 expression on ALL cells from 24 patients by flow cytometry. To identify GPI-defective cells we also analyzed the GPI-anchored CD55 and CD66c. Of the 24 cases, 19 expressed CD52. In 11 cases clear CD52-CD55-CD66c- GPI-defective subpopulation (median 0.02%, range 0.01-25.8%) were demonstrated. We evaluated the *in vivo* activity of ALM against ALL, and the relevance of the GPI-defective subpopulations, in a preclinical model of human ALL. Two cases were selected: BV (4.0% $CD19^+CD52^-CD55^-$ cells, overall MFI 137) and CM (no detectable $CD19^+CD52^-CD55^-$ cells, overall MFI 616). NOD/scid mice were inoculated with primary BV or CM cells. From day 14 after inoculation, animals received daily injections of 250μg ALM (n=8) or saline (n=4). After 3 weeks bone marrow (BM) was analyzed by flow cytometry. BM of control treated animals engrafted with BV cells contained 80±20% leukemic cells, 95% were CD52+. BM of control treated animals engrafted with CM cells contained 69%±14% leukemic cells, 99% were CD52+. BM of ALM treated animals with BV or CM contained 75% (±5.9%) and 21% (±5.4%) leukemic cells, respectively. These cells were exclusively $CD52^-CD55^-CD66c^-$, demonstrating that ALM eradicated all $CD52^+$cells but selected GPI-defective subpopulations. As known from paroxysmal nocturnal hemoglobinuremia, GPI-defective cells are highly susceptible to complement mediated cytotoxicity (CDC) due to lack of the protective function of CD55. Since ALL cells recovered from ALM treated and control treated mice showed similar expression of CD20, we compared their susceptibility to rituximab (RTX) mediated CDC. In the presence of 10μg/mL RTX, 50% lysis of CD52 positive ALL cells required a 1:40 complement dilution while 50% lysis of CD52 negative GPI-defective cells required a 1:200 dilution. Similarly, in the presence of a 1:100 complement dilution, 2.5 μg/mL RTX mediated 10% lysis of CD52 positive ALL cells but 75% lysis of CD52 negative GPI-defective cells. To investigate the *in vivo* relevance of these findings we engrafted NOD/scid mice with CM or BV cells and administered RTX (250μg daily) or RTX plus ALM (250μg each daily). Administration of RTX alone resulted in only partial reduction of leukemic cells. However, combined administration of ALM and RTX resulted in complete remissions in all CM-engrafted animals, 75% of which were molecular remissions as demonstrated by RT-PCR, and in 4 of 5 BV-engrafted animals. In summary, ALM is highly effective against $CD52^+$ ALL cells but $CD52^-$ GPI-defective subclones may preexist and escape therapy. Since GPI-defective cells are highly susceptible to complement mediated lysis, combination treatment of ALM with antibodies directed against non-GPI anchored proteins, such as RTX is rational and likely to be synergistic.

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PLERIXAFOR TARGETS PRIMARY CHRONIC MYELOID LEUKAEMIA STEM/PROGENITOR CELLS AND ENHANCES THEIR SENSITIVITY TO TYROSINE KINASE INHIBITORS

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Despite recent advances in the treatment of chronic myeloid leukaemia (CML) with tyrosine kinase inhibitors (TKIs; imatinib, dasatinib, nilotinib) the majority of patients have persistent molecular disease. This is believed to result from the failure of TKIs to target the most primitive quiescent CML stem cells. There is increasing evidence for chemoresistance via the SDF1/CXCR4 axis in CML and other leukaemias. In CML, BCR-ABL interferes with SDF1-induced migration to the bone marrow niche by down-regulating CXCR4 expression. This is restored by TKIs, promoting the survival of quiescent CML cells by increasing their migration to the bone marrow niche (Jin *et al.*, 2008). Plerixafor (Mozobil®; formerly AMD3100; Genzyme Corporation, Cambridge, MA, USA) is a CXCR4 antagonist and is currently used to mobilize $CD34^+$ haematopoietic progenitor cells in humans. *in vitro* studies in CML cell lines have shown that plerixafor inhibits chemotaxis towards SDF1, resulting in increased susceptibility of BCR-ABL-positive cells to TKIs (Dillman *et al.*, 2009). The aim of this study was to determine if plerixafor reverses the quiescence of primary $CD34^+$ CML stem/progenitor cells *in vitro*, rendering these primitive cells sensitive to TKIs. We investigated the *in vitro* response of primary chronic phase (CP) CML $CD34^+$ cells, and the BCR-ABL^+ cell line BV-173, to plerixafor. Total CP CML $CD34^+$ cells were assessed for cell viability (trypan blue exclusion method), proliferation (Brdu incorporation), apoptosis (Annexin V/viaprobe), cell migration (transwell plates), and colony forming potential (colony forming assays). Cells were cultured in both serum free medium supplemented with growth factors (SFM+5GF; IL-3, IL-6, G-CSF, Flt-3L, SCF) and in stromal co-culture assays using M2-10B4 and SL/SL fibroblasts. Plerixafor had no effect on viable cell counts or proliferation of CP CML $CD34^+$ cells up to 3 days following treatment. However from day 6 onwards we observed a dose-dependent reduction in cell numbers in the presence of plerixafor, for cells cultured SFM+5GF or SFM+G-CSF. Annexin V/viaprobe staining demonstrated that plerixafor did not induce apoptosis in these cells. Plerixafor inhibited migration of both primary CML and BV173 cells towards SDF1 and reduced chemotaxis to levels comparable with that of random spontaneous migration. In stromal co-culture experiments, we observed a trend towards reduced viability in cells treated with a combination of plerixafor and imatinib when compared to imatinib alone. After 6 days treatment with imatinib, primary CML $CD34^+$ cells showed decreased colony forming potential ($P<0.001$) and this decrease was enhanced significantly by combination treatment with plerixafor ($P<0.05$). Surface expression of CXCR4 on BV173 and primary $CD34^+$ CML cells was also significantly upregulated after treatment with imatinib ($P<0.05$ and $P<0.01$, respectively). However plerixafor overcomes this response and inhibits CXCR4 expression in a dose-dependent manner, reaching maximum inhibition at 1.0 μg/mL. Plerixafor's ability to inhibit CXCR4 expression and consequently SDF1-induced migration makes this drug an attractive therapeutic agent in the treatment of CML and other leukaemias. We hypothesize that plerixafor could be used to sensitize CML stem cells to TKIs, and be used in combination with TKIs to reduce minimal residual disease and in some cases disease elimination.

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SAFETY AND EFFICACY OF SECOND-LINE BOSUTINIB (SKI-606) IN IMATINIB RESISTANT OR INTOLERANT CHRONIC PHASE (CP) CHRONIC MYELOID LEUKEMIA (CML)

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Background. Bosutinib is an oral Src/Abl kinase inhibitor, with minimal inhibitory activity against PDGFR or c-kit. *Aims.* A phase 1/2 study of CP CML patients (pts) who have failed treatment with imatinib (IM) is ongoing. *Methods.* Pts received 500 mg bosutinib orally, daily, and were followed for safety and efficacy. *Results.* We report preliminary data for 299 pts, 70% CP IM-resistant, 30% CP IM-intolerant, with a median follow-up from start of bosutinib of 16.8 (0.56 - 43.8) months. Median age was 52 (18 - 91) years, and prior treatments other than IM included interferon (92 pts) and stem cell transplant (8 pts). Of 133 pts evaluable for hematologic response, 108 (81%) achieved complete response (CHR). 137/220 (62%) evaluable pts attained a major cytogenetic response (MCyR), of which 109 (50%) were complete. Of 155 pts evaluable for molecular response, 75 (48%) reached a major molecular response, 46 (30%) of which were complete. Responses by outcome after IM are summarized in the table. 20 different mutations were detected at baseline in 45/99 (45%) pts tested. CHR was achieved in 87% of 23 evaluable pts with mutations and in 93% of 28 evaluable pts with no mutation. MCyR was attained in 68% and 56% of 41 and 39 evaluable pts with and without mutations, respectively. The most common adverse events were gastrointestinal (nausea, vomiting, diarrhea), typically grade 1/2, manageable and improving spontaneously after 3 - 4 weeks. Grade 3/4 non-hematologic toxicities (>5% of pts) were diarrhea (8%) and rash (8%). A single pt with a history of left ventricular ejection fraction dysfunction on IM experienced grade 3 pleural effusion. One pt with a history of arrhythmias, myocardial infarction and pacemaker placement experienced grade 3 prolonged QTc interval while on bosutinib. The most frequent grade 3/4 hematologic abnormalities were thrombocytopenia (23%), neutropenia (14%) and anemia (9%). Additional common grade 3/4 laboratory abnormalities included hypermagnesemia (11%) and increased ALT (10%). 91 pts (30%) required at least one dose reduction. Median daily dose was 480 mg for IM-resistant pts and 401 mg for IM-intolerant pts. 59 pts (20%) discontinued bosutinib due to toxicity. *Summary/Conclusions.* Bosutinib is effective in CP CML pts who have failed IM. Clinical responses are achieved in pts with a wide variety of Bcr-Abl mutations except T315I. Bosutinib is well-tolerated with minimal hematologic toxicity.

Table.

	IM-Resistant N (%)	IM-Intolerant N (%)
Hematologic Response		
Evaluable*	92	41
Complete	74 (80)	34 (83)
Cytogenetic Response		
Evaluable*	164	56
Major	98 (60)	39 (70)
Complete	77 (47)	32 (57)
Molecular Response		
Evaluable*	109	46
Major	54 (50)	21 (46)
Complete	30 (28)	16 (35)

* Pts without CHR, CCyR or CMR at baseline, and with at least one post-baseline assessment for respective response

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LONG-LASTING P210 PEPTIDE VACCINE TREATMENT IN CHRONIC MYELOID LEUKEMIA PATIENTS: SAFETY PROFILE AND CLINICAL RESULTS

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Background. In chronic myeloid leukemia (CML) BCR-ABL derived p210 protein is a unique antigen for an immune target therapy and we have designed two p210-derived peptide vaccines CMLVAX100 (5 b3a2 peptides) and CMLVAXb2a2-25 (1 b2a2 peptide) for improving minimal residual disease (MRD) surviving conventional treatment. Pivotal studies have shown that vaccine treatment may induce a reduction of residual CML. Currently, two multicenter clinical trials are ongoing to better define the role of peptide vaccines in a larger cohort of patients with MRD during imatinib. Up to date little is known about safety profile and overall results of long lasting immune therapy with p210-derived peptide vaccines. *Aims.* of the study: Evaluation of safety and efficacy of p210-derived peptide vaccines in CML patients treated for a long period of time. *Methods.* CML patients with MRD during imatinib or interferon alpha (IFN-α) received either CMLVAX100 or CMLVAXb2a2-25 as part of phase I study vaccine trials conducted in our institution. Vaccine schedule included an immunization phase lasting 3 months eventually followed by a maintenance treatment every 3-6 months. Primary endpoint was the evaluation of immune and disease response after immunization phase. Among a total of 31 CML vaccinated patients, 14 continued maintenance treatment for a median of 4 years and are evaluated in this report. *Results.* At beginning of vaccinations 9/14 patients were in late CP (median time from diagnosis 56 months); 12/14 patients were under imatinib treatment for a median time of 24 months (range 6-84); 1/14 was under IFN-α since 84 months and 1/14 was not receiving any CML treatment. All 14 patients were vaccinated in complete cytogenetic response (CCyR) with clear evidence of MRD (median BCR-ABL/ABL ratio 0,8 range 0.03-2.27). They received a median of 17 vaccinations each (range 13-22) during a median time of 49.5 months (range 36-67) from first vaccination. Vaccinations were very well tolerated and no patient showed any clinical toxicity other than mild local redness and itching. *In vitro* vaccine-induced immune response was stably documented in 8/14 patients, while 4/14 patients showed a temporary response and 2/14 patients had no evidence of response. During vaccinations 11/14 patients showed a reduction of MRD. At present time 13/14 patients are still in CCyR after a median time from diagnosis of 124 months (range 60-216); one patient lost CCyR after 70 and 39 months from starting imatinib and vaccinations, respectively. At last follow-up patients' MRD status is as follows: 5/11 patients on imatinib are in complete molecular response (CMolR), while 6/11 maintain MRD (median BCR-ABL/ABL ratio 0,7 range 0,05-1,5); the patient on IFN-α achieved CMolR despite reducing treatment dose; a patient achieved CMolR with vaccine therapy only. *Conclusions.* p210-derived peptide vaccines are safe and may be given for a long period of time without evidence of toxicity. Long lasting treatment resulted in the achievement of a stable CMolR in 7/14 CML patients. As well importantly 6/7 patients with persistent high level of MRD (median BCR-ABL/ABL ratio 0,7) maintain a CCyR after a median time of 43 months from starting vaccinations.

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NILOTINIB INDUCES RAPID AND DURABLE RESPONSES WITH 24-MONTH MINIMUM FOLLOW-UP IN PATIENTS WITH IMATINIB-RESISTANT OR -INTOLERANT CHRONIC MYELOID LEUKEMIA IN BLAST CRISIS (CML-BC)

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Background. Nilotinib is a highly potent and selective inhibitor of BCR-ABL approved for the treatment of chronic phase (CP) and accelerated phase (AP) Philadelphia chromosome positive (Ph^+) CML in adult patients resistant to or intolerant of prior therapy, including imatinib. *Aims.* Here we present results from patients with imatinib-resistant and -intolerant Ph^+ CML-BC treated with nilotinib in the phase 2 registration trial. *Methods.* Patients were treated with nilotinib 400 mg twice daily. Primary endpoint was confirmed hematologic response (HR). *Results.* Patients (N = 136) with imatinib-resistant (82%) or -intolerant (18%) CML-BC (105 myeloid blast crisis [MBC]; 31 lymphoid blast crisis [LBC]), were treated with nilotinib and followed for a minimum of 24 months. In patients who had available baseline data, baseline BCR-ABL mutations were detected in 26/86 (30%) patients with MBC and 17/28 (61%) with LBC; T315I mutations were detected in 1 (1%) patient with MBC and 2 (7%) with LBC. Clonal evolution occurred in 73 (54%) patients. Stable or progressive disease was the best prior response to imatinib in 62 (46%) patients, with only 14 (10%) achieving prior complete HR (CHR) and 15 (11%) achieving major cytogenetic response (MCyR) on imatinib. With nilotinib therapy, 25 (24%) patients with MBC and 6 (19%) with LBC achieved a confirmed HR (Table). CHR was attained by 14 (13%) and 4 (13%) patients with MBC and LBC, respectively. Responses were rapid and durable. Median time to HR was 1 month and median duration of HR was 26 months (range, 1.25 - 29.08) for patients with MBC; 60% of these patients maintained HR at 24 months. For patients with LBC, the median duration of HR was 3.6 months and no patient maintained HR at 24 months. Nilotinib resulted in MCyR and CCyR in 40 (38%) and 31 (30%) patients with MBC and 16 (52%) and 10 (32%) with LBC, respectively. In patients with MBC, median time to MCyR was 2 months and median duration of MCyR was 11 months (range, 0.03 - 29.08). MCyR was maintained in 44% of patients at 24 months. Median duration of MCyR was 3 months for patients with LBC and no patient with LBC maintained MCyR at 24 months. Survival was 42% at 12 months and 27% at 24 months; 12 patients with MBC and 2 patients with LBC underwent transplants following nilotinib therapy. Nonhematologic adverse events were generally mild and manageable and similar to those observed in patients with Ph^+ CML-CP. *Conclusions.* Nilotinib is an effective therapy in patients with imatinib-resistant or -intolerant Ph^+ CML-BC, generating rapid and durable responses for patients with MBC. Nilotinib exhibited a favorable safety profile in patients with Ph^+ CML-BC and no new safety concerns were observed with 24 months minimum follow-up.

Table. Response rates in patioents with CML-BC treated with nilotinib.

Response	Overall (N = 136)	MBC (n = 105)	LBC (n = 31)
HR, n (%)	31 (23)	25 (24)	6 (19)
CHR, n (%)	18 (13)	14 (13)	4 (13)
MCyR, n (%)	56 (41)	40 (38)	16 (52)
CCyR, n (%)	41 (30)	31 (30)	10 (32)
12-month OS, %	42	44	35
24-month OS, %	27	32	10

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EARLY CYTOGENETIC AND MOLECULAR RESPONSES AND PHARMACOKINETIC OF DASATINIB AS A FIRST LINE THERAPY IN NEWLY DIAGNOSED CHRONIC PHASE CML PATIENTS: FIRST ANALYSIS OF THE OPTIM DASATINIB TRIAL

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Background. Dasatinib 100 mg QD is approved as second line therapy in patients with chronic myelogenous leukaemia in chronic phase (CP-CML) after imatinib failure or intolerance. As suggested in second line therapy, efficacy and tolerance of dasatinib first line may be driven by pharmacokinetic parameters. We initiated an optimization study based on the monitoring of dasatinib plasma levels (Cmin and Cmax) administered as front line therapy in patients with newly diagnosed CP-CML. *Methods.* Patients aged 18 years old or more were eligible if they were newly diagnosed with CP-CML without having being exposed to tyrosine kinase inhibitors. Dasatinib was initiated at 100 mg QD. The pharmacokinetic (PK) evaluation (Cmin and Cmax) was performed after 7 to 10 days of therapy and every 3 months thereafter. Dasatinib dose adaptation was required in patients with a Cmin over 5nM and randomized in the treatment adaptation arm. All other patients were treated with dasatinib 100 mg QD. *Results.* We report the results of the first 57 patients included in the trial. Median age was 51 years (19-78) with a sex ratio M/F of 1.65. 52.9% of the patients presented with a low Sokal score, 29.5% and 17.6% with an intermediate and high score. Median Cmin and Cmax values were 2.3 nM (range 0.2-18.7) and 113.1 nM (range 20.5-261) respectively at first PK analysis. The median Cmin value was significantly lower in patients with age <50y compared to patients >50y (1.6 nM *vs.* 3.3 nM, P=0.007). By contrast, the median Cmax value was comparable in both age groups (119.8 nM *vs.* 92.2 nM, P=0.78). Efficacy was analyzed on the 38 patients with at least 3 months follow-up and on the 20 patients with 6 months follow-up. The complete cytogenetic response (CCR) rates at 3 and 6 months were 68% (26 out of 38) and 85% (17 out of 20) respectively. The major molecular response (MMR) rates at 3 and 6 months were 13.1% and 60%. Of note, the 3 patients not in CCR at 6 months had the highest Sokal score including two patients in cytogenetic failure (Sokal score values 2.5 and 1.8). Median PK values at months 3 and 6 were not statistically different compared to initial PK values, suggesting a stable exposure to the treatment with time. With a median follow-up of 4.3 months, only one pleural effusion was observed (1.6%). Haematological grade 3 and 4 toxicities in patients treated for at least 3 months included 4 episodes of neutropenia and 2 episodes of thrombocytopenia (4 patients out of 38, 10.5%). *Conclusions.* Dasatinib 100 mg QD as first line therapy in CP CML provided high rates of MMR (60%) and CCR (85%) as early as 6 months after treatment initiation. Pharmacokinetic parameters of dasatinib were different in aged patients. A complete pharmacokinetic analysis with correlations to response and tolerance will be presented on more patients with a 6 months follow-up.

Myeloproliferative disorders - Clinical

1140

PATTERNS OF SURVIVAL AMONG 7,249 PATIENTS WITH MYELOPROLIFERATIVE NEOPLASMS DIAGNOSED IN SWEDEN 1973-2003

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Background. Available data on survival patterns among patients diagnosed with myeloproliferative neoplasms (MPN) are inconclusive. Based mostly on smaller clinical series, patients diagnosed with primary myelofibrosis (PMF) have a substantially reduced life expectancy and polycythemia vera (PV) is associated with an inferior survival in most, but not all, studies. In essential thrombocythemia (ET), the situation is even more controversial with reports stating that life expectancy is not significantly affected by the disease while other investigators report that ET must be considered a serious disease that significantly decreases life expectancy. *Aims.* To establish patterns of survival in MPN patients in a population-based setting in Sweden. *Methods.* Using the nationwide Swedish Cancer Registry, we assessed patterns of survival among all MPN patients (n=7,249) reported 1973-2002 with follow-up data until 2003. Relative survival ratios (RSR) and excess mortality rate ratios (EMRR) were computed as measures of survival. *Results.* A total of 7,249 MPN patients were identified (PV n=3,784, ET n=1,987, PMF n=852 and MPN not otherwise specified (MPN NOS) n=626); 48% were males and the median age at diagnosis was 70 years. There was a significant overall excess mortality in patients with MPN, reflected in a 1-year RSR of 0.89 (95% CI 0.89-0.90), a 5-year RSR of 0.73 (0.71-0.74) and a 10-year RSR of 0.57 (0.55-0.59). For the different subtypes the 5-year and 10-year RSRs were 0.81 (0.79-0.83) and 0.64 (0.62-0.67) for PV, 0.75 (0.72-0.78) and 0.64 (0.60-0.68) for ET and 0.39 (0.35-0.43) and 0.22 (0.19-0.27) for PMF, respectively (Figure). Survival of patients with MPN has improved significantly over time with an EMRR of 0.55 (0.49-0.62) in 1980-1989 and 0.21 (0.17-0.25) in 1990-1999 using the calendar period 1973-1979 as a reference. Older age at MPN diagnosis was consistently associated with a poorer survival. For example, 10-year RSR for patients <50 years was 0.84 (0.81-0.87) as compared to 0.31 (0.24-0.41; P<0.001) in those >80 years. Females had a better survival than men, EMRR 0.73 (0.67 to 0.80). *Summary/Conclusions.* In this large population-based study including over 7,000 MPN patients, we found all MPN subtypes to have a significantly decreased life expectancy compared to the general population. ET patients were observed to have a poorer survival than patients with PV up to 10 years after diagnosis. A certain misclassification of ET as PMF, especially during earlier years, may have contributed to a reduction in survival rates in the ET group. Survival of MPN patients improved over time in all age groups. This may reflect in part the earlier establishment of a MPN diagnosis in more recent years but also the introduction of more effective treatment strategies and better supportive care. We are now analyzing causes of death in the cohort aiming at a better understanding of the excess mortality which may have implications for the clinical management of these disorders.

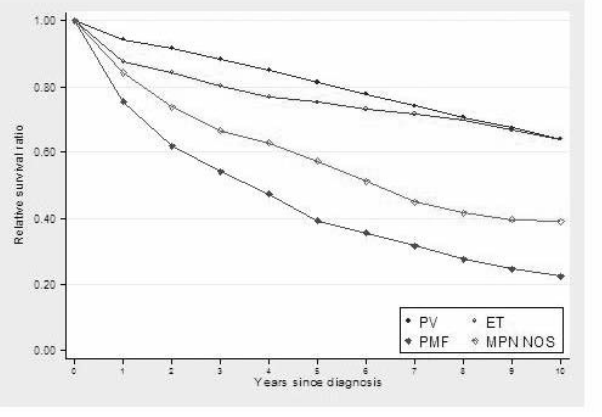

Figure. Cumulative relative survival stratified by subtype.

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THE JAK2 46/1 (GGCC) HAPLOTYPE IS ASSOCIATED WITH PRIMARY MYELOFIBROSIS INDEPENDENTLY OF V617F MUTATIONAL STATUS, DISEASE CHARACTERISTICS OR PROGNOSIS

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Background. A germline haplotype block, including most of JAK2, named 46/1, or "GGCC", has been shown in three studies (Jones AV *et al.*; Olcaydu D *et al.*; Kilpivaara O *et al.*; 2009) to be strongly associated with JAK2V617F myeloproliferative neoplasms (MPN). Additional studies showed significant association also with JAK2 exon 12-mutated polycythemia vera, JAK2-wild type essential thrombocythemia or primary myelofibrosis (PMF), suggesting an increased risk of MPN regardless of the V617F mutation. Whether this haplotype also conferred specific clinical characteristics is still under debate. *Aims.* To evaluate the association of JAK2 46/1 haplotype with JAK2V617F mutational status, clinical characteristics and prognosis in series of 202 PMF patients.*Methods.* Screening for 46/1 haplotype was performed in granulocyte DNA using a tag rs12343867SNP (T to C shift), that is in complete linkage disequilibrium with 46/1, in a RT-PCR allelic discrimination assay. The C allele stands for 46/1 haplotype. JAK2V617F allele burden was measured by RTQ-PCR. The study was approved from IRB and patients gave consent to use of their DNA samples. *Results.* Median follow-up was 65.4 months; 42 patients died (20.8%), and leukemia transformation occurred in 25 patients. Patients in the four IPSS risk categories accounted for 36.1%, 22.5%, 23.7% and 17.7% of the 169 who were evaluable. The frequency of C allele was significantly higher in the whole series of PMF patients compared to control population (0.386 *vs.* 0.266; P=0.0002). Such difference was largely due to the category of JAK2V617F mutated patients (C allele frequency, 0.413; P<0.0001 *vs.* controls), while in case of JAK2V617F negative patients a bordeline significance was measured (P<0.05). In JAK2V617F mutated patients with a V617F burden lower than 25% the frequency of C allele was 0.333, similar to JAK2 wild-type population (P=0.434), while a C allele frequency of 0.416 was found in those with greater than 25% V617F allele (P<0.0001 *vs.* controls). Furthermore, the frequency of C allele increased from 0.333 to 0.500 (P<0.0001, chi-square test for trend) in patients divided in JAK2V617F burden quartiles. We found no difference regarding hematological parameters, large splenomegaly, constitutional symptoms, IPSS score, rate of transformation to leukemia or death, depending on the patient rs12343867 genotype. Also, there was no difference in overall survival in the three rs12343867 genotypes even after stratification of the patients according to their JAK2V617F mutational status. *Conclusions.* Present data confirm the significant association of 46/1 haplotype with PMF described in earlier studies, and point to a major contribution of V617F-mutated patients. In line with a recent study from Mayo Clinic (Tefferi A *et al.*, Leukemia 2009) we observed that the C allele was over-represented in patients showing the highest V617F allele burden, thus underlining the contribution of UPD in the progressively increasing rate of 46/1 haplotype according to V617F allele burden. On the other hand, these results are at variance with the Mayo study where the TT allele was significantly associated with shortened survival, particularly in JAK2 wild-type patients. Additional studies are needed to fully clarify the prognostic role of 46/1 in PMF.

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MUTATIONS OF IDH1 AND IDH2 IN MYELOPROLIFERATIVE NEOPLASMS: ASSOCIATION WITH LEUKEMIC TRANSFORMATION

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Background. Essential thrombocythemia (ET), polycythemia vera (PV), and myelofibrosis (MF) are myeloproliferative neoplasms (MPN) characterized by clonal expansion of myeloid cells. The understanding of the molecular pathogenesis of MPN is mainly based on the identification of somatic mutations in the genes *JAK2*, *MPL*, and *TET2*. However, a significant number of patients lack any proof of clonality. More recently, mutations in isocitrate dehydrogenase 1 (*IDH1*) and 2 (*IDH2*) genes have been identified in myeloid malignancies resulting in the production of a potential oncometabolite (2-hydroxyglutarate). *Aim.* In our study, we sought to explore the presence of exon 4 mutations of *IDH1* and *IDH2* in a cohort of well-characterized MPN and acute myeloid leukemia cases secondary to MPN (sAML). *Methods.* Exons 4 of *IDH1* and *IDH2* were analyzed for mutations in 115 patients [PV, n=30; ET, n=30, MF, n=20; sAML, n=35] using a combination of denaturing high-performance liquid chromatography and DNA sequencing. Data on the mutation status of *JAK2* (V617F), *MPL* (W515L), and *TET2* (all exons sequenced) were available in all cases. In addition, all cases were analyzed on 250K single-nucleotide polymorphism (SNP) arrays that allow for genome-wide screening of copy-number alterations (CNAs) and uniparental disomies (UPDs). *Results.* In total, seven heterozygous *IDH* missense mutations were identified. Mutations were rare in MPN (n=1), whereas 17% (6/35) of sAML cases harbored *IDH1* (n=2) or *IDH2* (n=4) mutations. In sAML, both *IDH1* mutations affected the same single arginine residue (R132H and R132C, respectively); *IDH2* mutations were restricted to the amino acid residues R140 (n=3; R140Q) and R172 (n=1; R172K). The *IDH1* mutation in one MPN case (ET) was detected in an atypical position (E62K). This patient lacked further genomic aberrations or *JAK2/MPL/TET2* mutations. Although *JAK2* and *TET2* mutations in sAML were relatively frequent in our study (60% and 26%, respectively), additional genetic lesions were rare in the six *IDH* mutated sAML patients: in the one case with *IDH2* R172K mutation an additional *TET2* mutation in exon 10 was found (G1859W), whereas *JAK2* V617F was mutated in two patients with *IDH2* R140Q mutation. Of note, both *JAK2/IDH2* double-mutated cases had leukemic transformation from preceding PV; UPD in 9p including the *JAK2* locus was present in one of the cases. Our previous SNP-array study on sAML patients revealed CNAs in 65% of cases; 35% of samples showed complex genomic aberrations with up to 20 CNAs per patient (Stegelmann *et al.,* ASH 2009, Abstract #2608). Here, only one of the three exclusively *IDH* mutated sAML patients exhibited CNAs (del(7), trisomy 8, and trisomy 15) as assessed by SNP-chip analysis. Consequently, our data indicate that one third (2/6) of *IDH* mutated sAML patients lack additional genetic or genomic aberrations. *Conclusions.* Our study on 115 MPN/sAML patients revealed *IDH1* and *IDH2* mutations as a frequent genetic lesion in sAML secondary to MPN. Validation of the uncommon *IDH1* E62K mutation in ET is currently ongoing. Furthermore, our data suggest that *IDH1* and *IDH2* mutations may be implicated in a subset of sAML patients as an autonomous pathogenetic mechanism.

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EVIDENCE OF EFFICACY OF RAD001, AN INHIBITOR OF MTOR, IN A PHASE I/II STUDY IN PRIMARY MYELOFIBROSIS (PMF) AND POST POLYCYTHEMIA VERA/ESSENTIAL THROMBOCYTHEMIA MYELOFIBROSIS (PPV/PET MF)

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Background. JAK2V617F mutated cells display PI3K/Akt pathway activation. RAD001, a specific inhibitor of mTOR that is downstream of PI3K, inhibited JAK2V617F-pos cells reducing STAT5 phosphorylation, and prevented growth of MPN CD34+ cells (Bogani C *et al.*, ASH2009). *Aim.* We are conducting an investigator-initiated, multicenter phase I/II trial to test safety and efficacy of RAD001, as an off-label drug, in pts with PMF and PPV/PET MF, with intermediate/high risk score (Lille criteria) or requiring treatment because of progressing splenomegaly if in the low score. PMF and PPV/PET-MF were diagnosed according to WHO and IWG-MRT criteria, respectively. Phase I involved a 3+3 patients scheme in 3 sequential cohorts at 5.0, 7.5, and 10 mg once daily, orally, for 3 months. Phase II was a two-stage Simon design at MTD for 4 months. The protocol was approved by IRBs and patients provided informed consent. The trial is supported by Italian government AIFA agency. *Results.* MTD from Phase I was established at 10 mg daily, with no DLT; there were 2 major and 3 moderate responses, while 3 pts had no response (NR). In portion one of Phase II, 16 pts have been included and 10 are evaluable; data will be updated at the meeting. There were 6 PMF, 6 PPV- and 4 PET-MF. Nine pts were low and 7 intermediate Lille score at enrollment; according to IWG-MRT, 3 were low, 5 intermediate-I, 3 intermediate-II and 5 high risk score. In 2 cases therapy was discontinued because of patient's decision at day 60 without evidence of any AE >2 grade. Therapy was generally well tolerated; commonest toxicities were grade 2 mouth ulcers in 5 pts and grade 1/2 hypertrigliceridemia in 7 pts; one grade 3 asymptomatic hypopotassemia. Hematological toxicities were reversible one grade 2 and three grade 3 anemia, and one grade 2 neutropenia. Of the 8 patients who completed therapy a reduction of spleen size consistent with CR, PR or NR was obtained in 2, 5, and 1 pts, respectively. 4 of 5 patients with systemic symptoms had complete resolution, and all 4 pts reported disappearance of pruritus. One of 6 anemic pts had PR becoming transfusion-independent with Hb 108 g/L. A CR in platelet count was obtained in 1 of 6 pts, while none of 5 pts with leukocytosis had measurable response. Overall, there were 3 major, 4 moderate and 1 NR (EUMNET criteria), or 2 CI and 6 stable disease (IWG-MRT criteria); according to intention-to-treat analysis, there were one additional moderate and one NR (EUMNET) or 1 CI and one SD (IWG-MRT). Of the 5 JAK2V617F-pos pts one had reduction of allele burden of 24% (from 82% at baseline to 63%) while in the others minimal changes were measured. Circulating CD34+ were reduced of 18% to 68% the baseline value in 4 pts, in three there was an increase from 90% to 260% the baseline value, and in 1 patients no change. *Conclusion.* Preliminary results from trial Phase II suggest potential activity of RAD001 in pts with myelofibrosis.

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FIRST REPORT OF THE PHASE-I STUDY OF THE NOVEL ORAL JAK2 INHIBITOR SB1518 IN PATIENTS WITH MYELOFIBROSIS

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Background. SB1518 is a potent inhibitor of JAK2 (IC_{50}=22 nM) and the JAK2*V617F* mutant (IC_{50}=19 nM) implicated in the pathogenesis of myeloproliferative neoplasms including myelofibrosis. SB1518 is highly selective for JAK2 inhibition compared to JAK1 and JAK3 (58 and 24 fold, respectively) which inhibits proliferation of JAK-2-dependent human leukemia cell lines at nM levels and has antitumor activity in a nude-mouse model of JAK2-dependent (BaF3-JAK2*V617F*) leukemia. Based on these encouraging data, SB1518 was evaluated in subjects with advanced myelofibrosis in a Phase-I dose escalation study. *Aims/Methods.* To determine the safety, tolerability and PK/PD profile of SB1518 when administered orally once daily continuously in 28-day cycles and define the recommended phase-II dose. Plasma samples for full PK analysis were taken at various time points on days 1, 15, and 25-28 of cycle 1. *Results.* From August '08-January '10, 20 patients have been enrolled and treated at 5 dose levels from 100-600mg daily. Demographic data are available for 20 patients and safety data for 18. Median age was 58.5 (range 47-74) years with 60% males, median prior therapies was 1 (0-7), and 85% were JAK2 mutation-positive. The median time from diagnosis was 39 months and 80% had clinical splenomegaly (median 17.5cm below costal margin) at study entry. No dose-limiting toxicities (DLTs) were seen at the 100, 200, or 400mg dose levels (each n=3). At 600 mg, 2 of 4 patients experienced DLTs (Gr 3 nausea/diarrhea/fatigue requiring temporary drug interruption). Cohort expansion at 500 mg has enrolled 4 additional patients without any DLTs in cycle 1, although 3 required later dose interruptions due to diarrhea, elevated ALT and dizziness, respectively. The most common drug-related adverse events have been diarrhea 89% (11% Grade 3, only observed at 500 mg and 600 mg dose levels), nausea and vomiting 39% (6% grade 3 nausea only at 600 and vomiting all Grade 1/2), abdominal pain, fatigue at 22% each (11% Grade 3 only at 600), and dysgeusia and rash 17% (all Grade 1/2). The median treatment duration is 99.5 days (max 414+ days). Six patients have ceased SB1518, while 14 continue on treatment. Using 2006 IWG response criteria, confirmed clinical improvements have been seen in hemoglobin (transfusion independence), platelet, and splenomegaly categories and updated response data will be presented. SB1518 was rapidly absorbed with a Tmax of 3-6 hours and mean elimination half-life of 22-55 hours. Steady-state drug levels showed no accumulation over the studied period. Plasma AUC increased in a dose-related manner between 100 and 400 mg dose levels, with full data from 500 and 600mg dose levels pending. SB1518 achieved potentially pharmacologically active concentrations at the starting dose of 100 mg on day 1. *Conclusion.* SB1518 is well tolerated in cycle 1 at doses below 600 mg daily in patients with advanced myelofibrosis, and shows promising clinical activity. The 500mg dose is not optimally tolerated for chronic dosing beyond cycle 1, and so 400mg is the dose selected for evaluation in the ongoing phase-II studies in patients with myelofibrosis.

Hodgkin's lymphoma

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TWO CYCLES OF ABVD FOLLOWED BY INVOLVED FIELD RADIOTHERAPY WITH 20 GRAY (GY) IS THE NEW STANDARD OF CARE IN THE TREATMENT OF PATIENTS WITH EARLY-STAGE HODGKIN LYMPHOMA: FINAL ANALYSIS OF THE RANDOMIZED GERMAN HODGKIN STUDY GROUP (GHSG) HD10 STUDY

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Background. There has been an ongoing debate on the best treatment for patients with early favourable Hodgkin lymphoma (HL). Open questions include the choice between combined modality treatment or chemotherapy only, the number of chemotherapy cycles needed and the optimal radiation dose. The GHSG thus conducted a randomized study for patients with early-stage favourable Hodgkin lymphoma (HD10) in which these questions were addressed. *Methods.* HD10 was an international prospectively randomized multicenter trial comparing 2 and 4 cycles of ABVD as well as 20Gy or 30Gy involved field radiotherapy (IFRT) in a 2 x 2 statistical design. Between 5/1998 and 1/2003, a total of 1370 patients from 329 centers were randomized into four arms: 4 x ABVD + 30Gy; 4 x ABVD + 20Gy; 2 x ABVD + 30Gy; 2 x ABVD + 20Gy. All patients had their initial histology reviewed by a lymphoma expert panel. Documentation was complete in more than 99,1% of cases for this final analysis. *Results.* Patients were equally balanced for age, gender, stage, histology, performance status and risk factors between arms. There were significant differences in major toxicity (WHO grade III/IV) between 4 x ABVD and 2 x ABVD in the overall number of events (52% *vs.* 33%) including leukopenia (24% *vs.* 15%) and hair loss (28% *vs.* 15%). In terms of radiation dose, there also was a difference in toxicity between 30Gy and 20Gy IFRT (all events: 8.7% *vs.* 2.9%), dysphagia (3% *vs.* 2%), mucositis (3.4% *vs.* 0.7%). Complete remission was achieved in 97% of patients treated with 4 x ABVD, 97% with 2 x ABVD, 99% after 30Gy and 97% after 20Gy. With a median follow-up of 79 - 91 months, there was no significant difference between 4 x ABVD and 2 x ABVD in terms of overall survival at 5 years (OS: 4 x ABVD 97.1%; 2 x ABVD: 96.6%), freedom from treatment failure (FFTF: 93.0% *vs.* 91.1%) and progression free survival (PFS: 93.5% *vs.* 91.2%). For the radiotherapy question, there were also no significant differences between patients receiving 30Gy IFRT and those with 20Gy IFRT in terms of OS (97.6% *vs.* 97.5%), FFTF (93.4% *vs.* 92.9%) and PFS (93.7% *vs.* 93.2%), respectively. Importantly, there was also no significant difference in terms of OS, FFTF and PFS when all four arms were compared. *Conclusion.* Two cycles of ABVD followed by 20Gy IFRT is the new GHSG standard of care for Hodgkin patients in early favourable stages.

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DACARBAZINE IS AN ESSENTIAL COMPONENT OF ABVD IN THE TREATMENT OF EARLY FAVOURABLE HODGKIN LYMPHOMA: RESULTS OF THE SECOND INTERIM ANALYSIS OF THE GHSG HD13 TRIAL

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Background. Combined modality treatment consisting of 2-4 cycles of ABVD (adriamycin, bleomycin, vinblastine, dacarbazine) followed by involved field radiotherapy (IFRT) is regarded as standard of care for early favourable Hodgkin Lymphoma (HL). However, the impact of bleomycin and dacarbazine in this combination is unclear and has been questioned for years. *Aims.* To determine the minimum required cytotoxic drugs, the GHSG (German Hodgkin Study Group) HD13 study compared 2 cycles of ABVD (arm A) with a dacarbazine-deleted variant (ABV, arm B), a bleomycin-deleted variant (AVB, arm C), and a variant in which both, dacarbazine and bleomycin were deleted (AV, arm D). *Methods.* Between 01-2003 and 09-2009, 1710 patients were enrolled into the HD13 study. Due to more events (defined as progressive disease, relapse, or death) during a continuous safety analysis, arm D was closed early in 09-2005, and arm B in 02-2006. 323 patients are evaluable for the comparison A (n=167) *vs.* D (n=156), and 389 for A (n=198) *vs.* B (n=191), respectively. Due to these low case numbers, descriptive analyses were performed comparing response and event rates and Kaplan Meier estimates. *Results.* Patient characteristics were well balanced between arm A, B, and D. With regard to toxicity, less leukopenia (CTC grade 3 or 4) was observed with the AV regimen (13% *vs.* 18% for ABVD). With regard to efficacy, the comparison of arm A *vs.* B showed an inferior lymphoma control by the reduced regimen as indicated by the complete remission (CR) rate (97.5% *vs.* 95.8%) and more patients with progressive disease (PD, 1.0% *vs.* 3.1%). The 4 year estimate for freedom from treatment failure (FFTF [95%-CI]) was also inferior (93.5% [88.8% to 96.2%] *vs.* 84.5% [77.9% to 89.2%]). However, overall survival (OS) at 4 years was not substantially different (98.4% [95.0% to 99.5%] *vs.* 95.9% [91.6% to 98.0%]). For significance of differences see Table 1. Very similar results were observed for the comparison of arm A *vs.* D with a CR rate of 97.0% *vs.* 91.0%, and PD in 1.2% and 5.8% of all patients. Accordingly, the 4 year FFTF was inferior (92.3% [86.9% to 95.6%] *vs.* 75.3% [67.0% to 81.8%]), but not the OS (98.1% [94.1% to 99.4%] *vs.* 98.7% [94.8% to 99.7%]). *Conclusion.* Reduction of the ABVD regimen to ABV (arm B) or AV (arm D) results in a decreased CR rate and an increase of patients with progressive disease or relapse. Accordingly, the FFTF difference at 4 years is high with 9% (A *vs.* B) and 17% (A *vs.* D), each in favor of the standard arm. Fortunately, most of these patients were successfully salvaged by second line treatment and this poor PFS did not translate into an inferior OS. To summarize, dacarbazine cannot be omitted in the ABVD regimen without significant loss of efficacy. Final analysis of the HD13 study (arm A *vs.* arm C) will show whether bleomycin can be safely omitted in the ABVD regimen.

Table 1. Estimates and 95%-CIs for 4 year differences (Δ).

	FFTF	PFS	OS
A vs B (ABVD versus ABV)	Δ -9.0% [-15.8% to -2.2%] HR 2.26 [1.19 to 4.28] p=0.01	Δ - 9.8% [-16.3% to -3.3%] HR 2.76 [1.37 to 5.54] p=0.003	Δ -2.4% [-6.4% to 1.5%] HR 1.64 [0.54 to 5.02] p=0.38
A vs D (ABVD versus AV)	Δ -17.0% [-25.6% to -8.4%] HR 2.81 [1.51 to 5.24] p=0.0007	Δ -16.8% [-25.1% to -8.5%] HR 3.25 [1.63 to 6.46] p=0.0004	Δ 0.6% [-3.0% to 4.2%] HR 0.61 [0.15 to 2.54] p=0.491

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EVIDENCE OF CLINICAL ACTIVITY IN A PHASE II STUDY OF ORAL PANOBINOSTAT IN PATIENTS WITH RELAPSED/REFRACTORY HODGKIN LYMPHOMA (HL) AFTER AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANT (AHSCT)

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Background. Panobinostat is a pan-deacetylase inhibitor targeting epigenetic and non-epigenetic oncogenic pathways. *in vitro*, panobinostat decreases proliferation and induces apoptosis in HL cell lines at low nanomolar concentrations. In a Phase I study, promising activity was observed in patients with relapsed/refractory HL (EHA 2009, Abstract

#1064). *Aims.* The aim of this pivotal, open-label Phase II study is to evaluate the efficacy of panobinostat in the post-transplant relapsed/refractory HL population. *Methods.* This study with Simon optimal 2-stage design has completed enrollment. Oral panobinostat is administered at a dose of 40 mg three times per week, every week, in 21-day cycles. Dose delay and modification for management of adverse events (AEs) is allowed. CT/MRI scans are conducted after every 2 cycles for efficacy evaluation. The primary endpoint is objective response rate. Secondary endpoints include time to response (TTR), duration of response (DOR), progression-free survival, overall survival, and safety. *Results.* As of February 8, 2010, 129 patients have been enrolled and treated: median age 32 years [18-75]; 30% had >5 prior lines of therapy; median number of prior regimens was 4 [1-6]; median time to relapse after first AHSCT was 8 months; >83% had 1 or more post-transplant therapies prior to study drug; 29% did not respond to last prior medication regimen. 11 patients also received prior allogeneic transplant. In preliminary efficacy analysis, 112 patients had >1 post baseline CT/MRI result available or discontinued early - 74% had tumor reduction, 4% had early PD, and 3% discontinued prior to first efficacy evaluation (e.g., due to AE). 24 responses (3CR+21PR) have been reported. 17 of 24 responders continue on study and tumor burden continues to decrease even after achieving PR and following dose modification. Although the median DOR cannot yet be determined, 50% of responders have a DOR ranging from 12-64+ weeks, and among the responders 8 patients have maintained response for >6 months. TTR ranged from 4-30 weeks among the 24 responders. Median treatment duration for 129 patients is currently 120+ days [5-511+] and 54% of patients continue on treatment. Common drug-related Grade 1/2 AEs were diarrhea, nausea, fatigue, vomiting, anorexia, and dysgeusia. Common drug-related Grade 3/4 AEs were thrombocytopenia (64%), anemia (14%), and neutropenia (12%). The thrombocytopenia was manageable and reversible with dose hold and modification. *Summary.* Interim results from this pivotal study to evaluate oral panobinostat shows promising activity in post-transplant relapsed/refractory HL patients. Manageable and reversible thrombocytopenia is the major toxicity in these heavily pretreated patients. Analysis of patient samples for predictive biomarkers to panobinostat is currently ongoing. Updated efficacy and safety data will be presented at the meeting.

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INCREASED EXPRESSION OF CD4+CD25+FOXP3+ REGULATORY T CELLS PREDICTS POOR RESPONSE AND CORRELATES WITH EPSTEIN-BARR VIRUS PRESENCE IN REED-STERNBERG CELLS IN PATIENTS WITH CLASSICAL HODGKIN LYMPHOMA

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Introduction. Unlike most other human malignancies, the Reed-Sternberg (RS) malignant cells of classical Hodgkin lymphoma (cHL) are vastly outnumbered by the surrounding nonmalignant inflammatory cells. The mechanisms of how RS cells perpetuates in this inflammatory milieu remain controversial and the Epstein-Barr virus (EBV) has been shown to play a role in this immune evasion. EBV can be found latently infecting RS cells in approximately 50% of all cases. EBV can increase the migration of $CD4^+$ lymphocytes that also express CD25 and FOXP3, named regulatory T cells (Tregs), which are specialized $CD4^+$ T cells that inhibit effector $CD4^+$ and $CD8^+$ T cells. In cHL, an increased number of Tregs is associated with loss of EBV-specific immunity. *Aims.* In this study, we assessed the distribution and biological significance of $CD4^+/CD25^+/FOXP3^+$ regulatory T-cells (Tregs) in 38 patients with cHL and its correlation with EBV presence in RS cells. *Methods.* Tissue microarrays were constructed using diagnostic biopsies available in 38 cHL patients and stained with CD4, CD25 and FOXP3 antibodies. Quantification of Tregs was performed using automated slide scanning and image analysis and correlated to phenotypic and clinical parameters in uni- and multivariate models. All patients had locally extensive or advanced stage disease and underwent similar chemotherapy protocols. For the present study, only cHL patients whose histology could be confirmed and EBV-association established were studied. *Results.* From the 38 cHL patients selected for this study, 21 were classified as EBV related and 17 EBV non-related cHL. The expression of FOXP(3) alone or together with $CD4^+CD25^+$ was more common in the EBV related cHL group ($P<0.001$) and was associated with either refractory disease or early relapse disease (less than 1 year) ($P<0.001$ and $P=0.03$, respectively). In a Cox regression model, considering gender, age and stage, the amount of $FOXP3^+$ cells was of independent prognostic significance for treatment response. Additionally, advanced stage disease, unrelated to EBV status in the tumor, was associated with strong expression of Tregs ($P=0.01$). Overall survival and event free survival were not calculated due to the short follow-up period. *Conclusions.* This study demonstrates that Tregs expression correlates with EBV presence in RS cells, advanced stage disease and poor outcome. Further studies investigating the mechanisms in which EBV recruits Tregs to the tumor microenvironment and its impact on the clinical evolution of cHL will contribute not only to our understanding on the pathogenesis of cHL but also to the development of therapeutic strategies designed to manipulate Treg activity.

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EARLY FDG-PET SCAN CONFIRMS ITS PROGNOSTIC IMPACT ALSO IN LOCALIZED STAGE, ABVD TREATED HODGKIN LYMPHOMA PATIENTS

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Background. Hodgkin's lymphoma is a malignant diseases with the highest rate of cure particularly if diagnosed in early stage. Nevertheless a small proportion of patients with localized stage do not respond to therapy and progressed. *Aims.* We explored the predictive value on therapy outcome of an early evaluation of treatment response by 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) scan performed after two corses of ABVD in pts with localized Hodgkin's disease. *Methods.* From 2002, 232 new localized stage Hodgkin's lymphoma pts were consecutively admitted to ten Italian hematological centers on behalf of Intergruppo Italiano Linfomi. Pts with stage I-IIA according to Ann Arbor stage, independent of presence of bulky disease, were considered for the study. FDG-PET was mandatory at baseline, after two cycles and at the end of therapy. Mediastinal blood pool activity is recommended as the reference background activity to define PET positivity. We evaluated the progression free survival of pts starting from the time of diagnosis to relapse or progression of disease or last follow-up. No treatment variation based only on PET-2 results was allowed. *Results.* The median age was 33 years (13-78), 123 pts were female, 213 pts were stage II, bulky was reported in 68 pts. Two-hundred and seventeen pts were treated with combined modality and 15 pts were treated with chemotherapy alone. The FDG-PET performed after two cycles (PET2) was positive in 32 pts (14%): 17 (53%) progressed or relapsed and 15 remained in CR. By contrast 190/200 (95%) pts with a negative PET2 remained in CR. Thus the positive predictive value of a PET2 was 53% and the negative predictive value was 95%. The sensitivity and specificity of PET2 were 63% and 93%, respectively. Twenty-one pts showed disease progression within 12 months after having reached CR, 13/21 were PET2 positive. Eight pts died due to the disease, five were PET2 positive and three were PET2 negative. In univariate analysis negative FDG-PET performed after two cycles (p .0000), absence of bulky disease at diagnosis (.005) were statistically correlated with a better progression free survival. In multivariate analysis only PET2 was independently predictive of relapse/progression probability (p .000). With a median follow-up of 35 months (range 4-87) 224 pts are alive and 205 (88%) are free from progression. The 2-yr FFS probability for PET2 negative and for PET2 positive patients were 94% and 44% respectively (P:.000). *Conclusion.* This multicentric study confirms that FDG-PET scan performed after two courses of conventional standard dose chemotherapy was able to predict treatment outcome in early stage Hodgkin disease. Due to the large number of false positive PET2 in localized lymphoma we suggest new PET evaluation methods in this subset of pts.

SCT and immunotherapy

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VCAM1 RS1041163 POLYMORPHISM INFLUENCES G-CSF MOBILIZATION OF CD34+ CELLS

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Background. The amount of CD34+ cells mobilized from bone marrow (BM) to peripheral blood (PB) after G-CSF administration greatly varies among healthy donors. Constitutional differences in genes involved in the disruption induced by G-CSF of the interactions tethering CD34+ cells to the BM might be associated with the number of G-CSF mobilized CD34+ cells. *Aims.* To evaluate a possible association between polymorphisms in genes involved in adhesive and chemotactic interactions to retain CD34+ cells within the BM with the number of G-CSF mobilized CD34+ cells influencing in the CD34+ cells yield obtained/kg of donor in healthy donors. *Methods.* Twenty seven polymorphisms described in sixteen genes (*CXCL12*, *CXCR4*, *VCAM-1*, *VLA-4*, *G-CSF*, *CSF3R*, *CD34*, *ADRB3*, *CXCL2*, *CXCR2*, *CD44*, *Kit ligand*, *c-Kit*, *MMP-9*, *CTSG*, *GNAS*) were analyzed by allelic discrimination PCR in 112 healthy donors receiving G-CSF (filgrastim; 10 μg/kg; 5 days) in a single institution and in 107 blood donor's volunteers from the Regional Center for Blood Transfusion of Seville. Univariate and multivariate regression analysis were performed to assess potential association between SNPs and the end points of the study. mRNA was measured by real-time quantitative PCR for those genes with SNPs found to influence CD34+ cell count at fifth day and CD34+ cells yield/kg of donor. *Results.* A genetic variant in *VCAM1* and another in CD44 were associated with a lower and higher quantity of CD34+ cells in PB after G-CSF (P=0.018 and P=0.039, respectively), with the yield of the first apheresis in terms of CD34+ cells/kg of donor (P<0.001 and P=0.025, respectively), and with the total CD34+ cells yield (P<0.002 and P=0.012, respectively). G-CSF administration was associated with a complete disappearance of *VCAM1* mRNA expression in PB and it did not affect the expression of different genotypes in the genetic variant in *CD44*. Moreover, a genetic variant in G-CSF receptor (*CSF3R*) and in *CXCL12* were associated with a lower and higher number of G-CSF mobilized CD34+ cells (P=0.002 and P=0.018, respectively). G-CSF greatly diminished the expression levels of *CXCL12* genetic variant. Finally, of the two variants in *CXCR4*, expression level of *CXCR4* variant.2 was the most influenced by G-CSF (P=0.001 and P<0.001 for variant.1 and variant.2, respectively) and a genetic variant in *CXCR4* was associated with lower yield of CD34+ cells/kg of donor (P=0.025), being this genotype the only one not affected by G-CSF at expression levels. *Conclusions.* Genetic individual variability in, *VCAM1*, *CD44*, *CSF3R*, *CXCL12* and *CXCR4* seems to influence HPC mobilization. *VCAM1* appears as the most important factor among all the molecules involved in mobilization. These findings might be useful in planning mobilizing strategies.

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POLYMORPHISMS OF THE HEPARANASE GENE (HPSE) ARE ASSOCIATED WITH THE INCIDENCE AND SEVERITY OF GRAFT *VS.* HOST DISEASE AFTER ALLOGENEIC STEM CELL TRANSPLANTATION

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Background. Graft-versus-host disease (GVHD) is the most common cause of overall mortality after HSCT. Heparanase, endo-β-glucuronidase that specifically cleaves the saccharide chains of heparan sulfate proteoglycans, is involved in the process of inflammation and release of heparan sulfate-bound chemokines, cytokines and bioactive angiogenic factors that are main players in the development of GVHD. *Aims.* The aim of the study is to investigate the association of HPSE gene SNPs with the risk of post HSCT GVHD and transplantation outcome. *Methods.* Four hundred and fourteen patients with hematological malignancies and their HLA matched donors were included in the study. Genotyping of two SNPs rs4693608 and rs4364254 was performed using allele-specific amplification. *Results.* Assessment of heparanase gene SNPs among healthy persons demonstrated a significant correlation between HPSE gene SNPs (rs4693608 and rs4364254) and the expression level of heparanase. All possible HPSE genotype combinations were distributed into three groups (LR, MR and HR) correlating with low, intermediate and high heparanase expression levels. In the group of HSCT patients we found a highly significant correlation between these SNPs, their combinations and risk of acute GVHD. The cumulative incidence of acute GVHD, grade II-IV, was 54.4% (95% CI 44.7-66.2) in the recipient group HR, while in recipient groups MR and LR, the cumulative incidences were 40.5% (95% CI 33.2-49.4) and 24.9% (95%CI 17.7-34.9), respectively (P=0.0001). Moreover, discrepancy between recipient and donor in these SNPs combinations significantly affected the risk of acute GVHD. Genotype combination LR in patients exerted a protective effect against GVHD regardless of the donor genotype combinations (D3 group: LR-LR, LR-MR, and LR-HR pairs). Acute GVHD rates were highest when recipients possessed genotype combinations HR, while their donors possessed the MR or LR genotype combinations (D1 group: HR-MR and HR-LR pairs). The other combinations were associated with an intermediate risk (D2 group: MR-MR, MR-HR, MR-LR and HR-HR pairs). Cumulative rate of acute GVHD incidence was 71.2% (95% CI 58.2-87.0) in the D1 group, 41.5% (95% CI 34.4-50.1) in the D2 group, and 24.9% (CI 95% 17.7-34.9) in the D3 group (P< 0.00001). The multivariate analysis revealed a strong association of HPSE gene SNPs with the risk of acute GVHD and a statistically significant correlation with extensive chronic GVHD. The significant associations of heparanase gene SNPs with TRM and overall survival, observed by univariate analysis, appear secondary to the effect on acute GVHD incidence. Summary: The study demonstrated that discrepancy in HPSE gene SNPs between recipients and donors is more relevant for the risk of developing acute GVHD than the patients' genotype profile of HPSE gene SNPs. Our findings may imply the involvement of heparanase in the pathogenesis of GVHD. We speculate that secreted levels of cytokines and chemokines affected by heparanase are higher in patients possessing HR genotype in comparison to possessors of the MR and LR genotypes. Higher cytokine and chemokine signals originating from the patient activate donor T-cells and increase the risk of GVHD. This aggressive phenotype of donor T lymphocytes results in infiltration and destruction of patient tissues and GVHD development.

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RESISTANCE OF AGGRESSIVE MINOR CML BLAST SUBSET TOWARD BOTH IMATINIB AND NK-CELLS KILLING CAN BE REVERSED BY PGP- MODULATORS

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Background. Although very effective in chronic phase CML, imatinib (IM) is usually less effective in advanced CML since drug-resistant clones inevitably shortly emerge. Moreover, immunotherapy via NK-cells is also less efficient in advanced CML. We have found that at blast crisis CML, blasts of the same CML clone are heterogeneous, containing a small subset (1-3%) of blasts that are significantly more aggressive than the major malignant population (Differentiation. 76: 908-922, 2008). These minor subsets (MS) of blasts (both from patients and human CML-BC cell lines) display higher clonogenicity than the common subsets (CS) and exclusively overexpress the multidrug transporter P-glycoprotein (Pgp). *Aims.* To evaluate whether the MS blasts also exhibit differential resistance mechanisms toward IM and NK-cells mediated killing, we compared the two blast subsets for the level of resistance to IM and for their sensitivity towards NK-cells in relation to expression of a functional Pgp. *Methods.* Various CS and MS blasts of CML-BC were evaluated before and after a range of single-drug selections (IM, doxorubicin, vinblastine, or colchicine) in culture. Protein and drug-efflux activity levels of the Pgp (ABCB1) multidrug transporter were evaluated by Western blotting, flow cytometry measurements and by UIC2-shift assays. The anti-proliferative effects of imatinib were measured by MTT proliferation assay, in the absence or the presence of the Pgp-specific modulator, R-verapamil (R-VRP). The killing capacity of healthy donor NK-cells and the NK-cell line NK-92 toward various target CS and MS blasts was measured by standard cytolysis assay, in the absence or the presence of the Pgp-modulator, R-VRP. *Results.* While Pgp could not be detected on the cell surface of the CS blasts, Pgp is exclusively highly expressed in the MS blasts. Functional Pgp assays in the MS blasts indicated unequivocally that IM is a substrate for Pgp. As IM efficiently inhibited the proliferation of the CS blasts in dose-dependent manner, the proliferation rate of the MS blasts was essentially not affected. Similarly, the MS blasts were significantly less affected by NK cells than the CS blasts (2.2±0.2 -fold, P<0.001). Further analyses indicated that the Pgp-expressing MS blasts become mainly less sus-

ceptible to the TRAIL-mediated apoptotic killing pathway of NK cells. Moreover, after short drug selections Pgp activity levels were further elevated by 1-order magnitude in the MS blasts with subsequent similar increase in the resistance levels to IM and the other abovementioned cytotoxic drugs. Similarly, the drug-selected MS blasts resistance toward NK cells killing was further increased (2.6±0.6 -fold, P<0.001). However, both the anti-proliferative effect of IM on the MS blasts and their sensitivity to killing by NK cells could be restored by addition of the Pgp inhibitor R-VRP, in a dose-dependent manner. *Conclusions.* The existence of a minor 'pool' of CML blasts of both greater clonogenicity and high expression and activity levels of Pgp, apparently signify clonal evolution toward both increased malignancy and lower therapeutic sensitivity to both IM and NK cells. Moreover, this study suggests that combination therapies with Pgp-modulators might also be clinically effective in targeting the MS aggressive blast population.

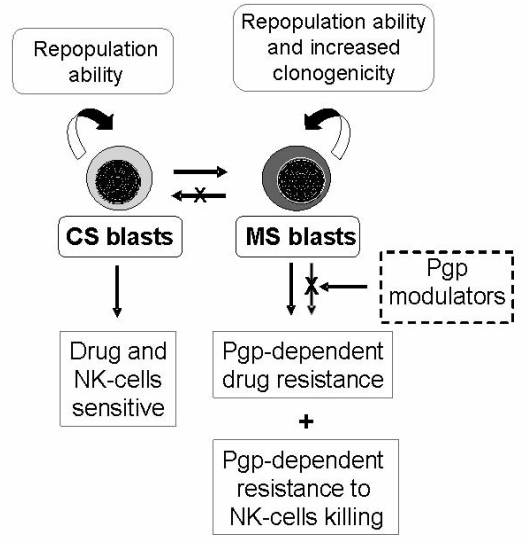

Figure. Differential properties and targeting of an aggressive CML blast subset.

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QUIESCENT LEUKEMIC STEM CELLS RESIDING AFTER TYROSINE KINASE INHIBITOR TREATMENT ARE NOT TARGETED BY ALLOREACTIVE T CELLS AND NK CELLS

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Tyrosine kinase inhibitors (TKI) like imatinib and dasatinib are the current treatment of choice for patients with chronic myeloid leukemia (CML). Most patients enter a complete remission during treatment, but recurrence of the disease is seen in the majority of patients upon discontinuation of treatment, and drug resistance may eventually occur illustrating that a fraction of leukemic stem cells is apparently capable of escaping the treatment. In these patients, allogeneic stem cell transplantation (allo-SCT) and application of immune effector cells may be a curative treatment. The aim of this study was to investigate whether the leukemic stem cells residing during TKI treatment are susceptible targets for immunological interventions using either alloreactive T cells or NK cells. $CD34^+$ positive CML cells were isolated from bone marrow and labeled with the fluorescent dyes CFSE or PKH to allow monitoring of single cell proliferation. CML cells were exposed to imatinib (1-100 μM) or dasatinib (0.01-50nM), and/or to $CD8^+$ alloreactive cytotoxic T lymphocyte (CTL) clones or donor-derived NK cells. The number, phenotype, and proliferative status of the CML cells residing after single and combined interventions were measured by quantitative flowcytometric analysis. In the absence of therapeutic interventions the majority of CML cells entered proliferation. However, a small population of $CD34^+$ CML stem cells residing in the non-dividing peak could be identified in all samples despite the addition of cytokines. TKI treatment resulted in efficient dose-dependent induction of cell death in the majority of cells. However, the population of quiescent $CD34^+$ CML stem cells was not affected and was even increased compared to the non-treated controls, indicating additional growth arrest of proliferating CML cells by TKI treatment. Next we tested the capacity of different HLA-A2 restricted $CD8^+$ CTL clones to kill non-treated or imatinib or dasatinib treated CML cells. Whereas the proliferating CML precursors were efficiently lysed, the population of quiescent stem cells was capable of withstanding CTL exposure. Detailed phenotypic analysis revealed significant downregulation of HLA-A2 and several adhesion molecules on these quiescent cells, probably resulting in the inability of these target cells to form a high avidity interaction with the T cells. Attempts to overcome the impaired HLA expression by treatment with interferon gamma or exogenous peptide loading were not successful. Next, we tested whether the impaired HLA class I expression on the quiescent population increased their susceptibility to NK cells. However, although the proliferating CML cells were efficiently targeted by the NK cells, the population of quiescent cells was not affected. In conclusion, TKI treatment results in selection of a population of quiescent leukemic stem cells showing cross-resistance to alloreactive T cells due to the impaired expression of molecules necessary for the formation of a high avidity interaction like HLA and adhesion molecules. Moreover, despite the impaired HLA class I expression, these cells were also not targeted by NK cells. These data indicate that TKI treatment does not act synergistically with immunological interventions. Furthermore, the anti-proliferative effect of tyrosine kinase inhibitors may potentially even hamper the potentially curative immune response after allo-SCT.

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VACCINATION WITH DENDRITIC CELLS LOADED WITH APOPTOTIC BODIES (APO-DC) OF AUTOLOGOUS LEUKEMIC CELLS INDUCES IMMUNOLOGIC RESPONSES IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

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Background. We previously demonstrated that dendritic cells (DC) that have endocytosed apoptotic bodies of autologous leukemic cells (Apo-DC) can boost antileukemic T cell responses *in vitro*. *Aims.* To study the safety and induction of immunity of Apo-DC vaccination in CLL patients. *Methods.* In this pilot study, sixteen patients with slowly progressing CLL, from whom informed consent had been obtained, were injected intradermally five times in 14 weeks with a mean of 16x106 ex-vivo generated Apo-DC. DC were generated from immunomagnetically enriched monocyte precursors, cultured *ex vivo* with GM-CSF and IL-4 and loaded with autologous apoptotic leukemic cells as antigen source. The patients were accrued stepwise to three different cohorts, receiving Apo-DC alone (n=5), Apo-DC + GM-CSF (n=5), or Apo-DC + GM-CSF + low-dose cyclophosphamide (n=6). Clinical and immune effects of the vaccination were evaluated at regular time intervals for 1 year. After 1-year follow-up, vaccination was re-initiated on a monthly schedule in five clinically stable patients belonging to cohorts II and III. A vaccine-induced immune response is defined as a ≥ 2-fold increase compared to pre-immunization values in either proliferation or ELISpot assay. Levels of regulatory T cells (Tregs) were also evaluated as well as secreted cytokines in the proliferation assay supernatants. CD107 cytotoxic assay was also performed. *Results.* To date, all 16 patients have completed the immunization protocol. No signs of autoimmunity were detected and only mild injection site reactions were noted, mainly associated to GM-CSF administration. Vaccine-induced immune responses were noted in 10/16 evaluable pts (2/5 in cohort I, 3/5 in cohort II and 5/6 in cohort III) and accompanied by high levels of IL-2, IL-5, IL-10 and IFN-γ in the proliferation assay supernatants. No clinical objective remissions were observed. Median time to progression was 14 months in immune responders *vs.* 8 months in non-immune responders (n.s.). The normalized frequency of regulatory T cells (Tregs) in 1-year follow-up was significantly lower in immune-responders *vs.* non-immune responders (P<0.0001). The presence of $CD8^+$, $CD4^+$ and CD16+CD56dim cells degranulating (CD107 cytotoxicity assay) in the presence of leukemic cells could be detected in almost all patients upon vaccination, with CD107-positive cells particularly found in cell aggregates (CLL cells fusing with T cells) as verified by confocal microscopy. *Summary/Conclusions.* The results show that immunization of patients with tumor-loaded DC induces specific immune responses in CLL patients especially when combined with GM-CSF and low-dose cyclophosphamide. Our data also confirmed that low levels of Treg are associated with the probability of inducing an immune response. This therapeutic approach shall be explored further in CLL patients without emerging need of chemotherapy.

Developmental biology

1155

ETO2 CONTROLS HEMATOPOIETIC STEM CELL EXPANSION VIA THE NERVY HOMOLOGY DOMAIN 1

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Background. Hematopoietic stem cells (HSCs) self-renew *in vivo* to sustain the life-long production of blood cells. However, HSCs rapidly decline in culture. Although some of the genes controlling self-renewal have been identified, overall, mechanisms governing HSC maintenance and self-renewal remain to be elucidated. We recently show that SCL (stem cell leukemia), a bHLH transcription factor, controls HSC quiescence and long term competence. Using a proteomics approach to identify components of the SCL complex in erythroid cells, we and others identified the ETO2 co-repressor that limits the activity of the SCL complex via direct interaction with the E2A transcription factor. ETO2/CBFA2T3 is highly homologous to ETO/CBFA2T1 and both are translocation partners for AML1. *Aims.* The objective is to define ETO2 function in HSCs. *Methods.* Eto2 expression was defined by qRT-PCR of purified long term -HSC (LT-HSCs, CD34-Kit+Sca+Lin- or CD150+CD48-Kit+Sca+Lin-), short-term HSC (CD34+Flt3-Kit+Sca+Lin-) and lympho-myeloid progenitors (LMPP, CD34+Flt3+Kit+Sca+Lin-) and protein levels were assessed by flow cytometry analysis of these populations. Eto2 functional studies include overexpression using the MSCV vectors, and RNA interference using lentiviral delivery. Transduced HSCs were analyzed by transplantation. *Results.* We initially found that ETO2 is highly expressed in populations of cells enriched in ST-HSCs and LMPP, and at lower levels in LT-HSCs, correlating with ETO2 protein levels detected by flow cytometry. Next, we showed that shRNAs directed against ETO2 knocked down ET02 protein levels in the KSL population, causing a ten-fold decrease in this population after transplantation; this was associated with reduced short-term and long-term reconstitution in mice. Conversely, ectopic ETO2 expression induced a 100 fold expansion of LT-HSCs *in vivo* in transplanted mice associated with differentiation blockade in all lineages, suggesting that ETO2 overexpression overcomes the mechanisms that limit HSC expansion *in vivo*. Furthermore, this expansion was abrogated when NHR1 domain of ETO2 was deleted. Surprisingly, overexpression of ETO2 but not of the NHR1 mutant resulted in a 1500 fold expansion of the KSL and KSL150+48- *in vitro* over 4 weeks. At the cellular level, we found that ETO2 facilitates the G0/G1 as well as G1/S transitions in LT-HSCs, whereas the NHR1 mutant causes G1/S blockade associated with apoptosis in the KSL population. *Sumary/Conclusions.* In conclusion, we show that ETO2 is highly expressed in ST-HSCs and lymphoid progenitors, and controls their expansion by regulating cell cycle entry both at the G0/G1 and the G1-S transitions. In addition, ETO2 overexpression converts the self-renewal of maintenance into self-renewal of expansion in LT-HSCs.

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DNA DAMAGE FROM STALLED REPLICATION IN HEMATOPOIETIC STEM CELLS LEADS TO P53-DEPENDENT BONE MARROW FAILURE THAT PREVENTS LEUKEMIC OUTGROWTH

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Background. DNA damage can result in tumorigenic mutations, but concurrently activates tumor suppressor pathways that abrogate expansion of progenitors to prevent cancer. Although generally accepted, this general theory fails to explain why different DNA repair syndromes have distinct phenotypes. We previously showed that mice deficient for the DNA repair protein Ercc1 age prematurely including loss of the transiently amplifying progenitors in the bone marrow (BM). Ercc1 functions in concert with the Fanconi Anemia (FA) pathway in removal of interstrand cross links (ICL) at the replication fork, but Ercc1 is also required for nucleotide excision repair (NER) in actively transcribed genes. *Aim.* The aim of our study was to investigate how DNA damage causes bone marrow failure and which tumour suppressor system is involved in hematopoietic dysfunction and leukemia. *Methods.* Mice deficient for the interstrand crosslink (ICL) and nucleotide excision repair (NER) protein Ercc1 and mice deficient for NER only were compared and crossed with p53 or Ink4A/Arf deficient mice. Hematopoietic parameters were analyzed by flow cytometry and colony assays. In addition, transplantation experiments were performed to study leukemogenesis *in vivo*. *Results.* Comparison of hematopoietic parameters between NER-deficient mice subject to severe premature aging and Ercc1-deficient mice indicated that defective NER causes the aging-related small stature, but hardly affects hematopoietic progenitors. This indicates that hematopoietic stem- and progenitor cells (HSPC) are exquisitely sensitive to replication fork stalling. The sensitivity to a stalled replication fork predicts progressive degeneration dependent on cell replication. Analysis of the fraction HSC and proliferating multipotent progenitors within the lineage-negative, $Sca1^+$, $cKit^+$ (LSK) BM fraction showed that quiescent stem cells were indeed protected from decay in Ercc1-deficient mice. Next, Ercc1-deficient mice were crossed with p53 and Ink4A/Arf deficient mice. This showed that the aging-related small stature is independent of the p53 or Ink4A/Arf tumour suppressor loci. Instead, loss of p53, but not loss of the Ink4a/Arf locus, restored the LSK fraction and colony forming ability of Ercc1-deficient BM. Outgrowth of Ercc1-deficient mouse embryonic fibroblasts was restored by loss of either p53 or the Ink4A/Arf locus. Thus the FA phenotype of Ercc1-deficient mice can be explained by a specific sensitivity of HSPC to ICL, and to p53 activation at the stalled replication fork. To investigate whether p53 controls leukemogenesis of Ercc1-deficient BM, we transplanted BM of Ercc1-deficient, p53 heterozygote mice in wt recipients. Whereas mice transplanted with BM of mice heterozygous for p53 but proficient in Ercc1 remained healthy for at least 12 months, 80% of mice transplanted with Ercc1-deficient BM heterozygous for p53 died within 5-10 months after transplantation. In 4 out of 8 leukemia's analyzed, the second p53 allele was deleted as well. *Conclusion.* Together these results show that stalled replication caused by deficiency in the repair of ICLs results in a strong p53-dependent bone marrow senescence which protects from leukemic outgrowth.

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MEGAKARYOCYTES SUPPORT THE INTEGRITY OF BONE MARROW (BM) VASCULAR NICHES AND ATTRACT METASTATIC TUMOUR CELLS TO THE BM IN MURINE MODELS OF METASTASIS AND IN PATIENTS WITH CARCINOMA

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Background. Bone marrow (BM) vascular niches maintain and regulate physiological haematopoietic cells and are implicated as areas of preferential engraftment for metastasizing tumour cells. *Aims.* To investigate the role of megakaryocytes in regulating the vascular niche in BM metastasis we used B16-F10 melanoma in normal (C57Bl/6 wild-type) mice and in mice with severely reduced megakaryopoiesis (C57Bl/6 $thrombopoietin\ (TPO)^{-/-}$ mice). *Results.* In wild-type mice with intradermal B16 melanoma, mean platelet volume progressively increased from 18 days following tumour injection ($P<0.0001$), suggesting increased thrombopoiesis; the number and size of megakaryocytes and mean BM vessel diameter were also increased compared to controls (P's<0.01). The majority of BM sinusoids were surrounded by one or more large megakaryocytes, forming the megakaryocyte-vascular niche, with megakaryocytes tightly abutting the vascular endothelium. From day 15, metastatic mCherry-transduced B16 cells were visible closely associated with megakaryocytes in the BM vascular niche. B16 cells expressed several thrombopoietic factors, particularly VEGF, SCF, and IL11, as well as smaller amounts of IL6 and TPO. In $TPO^{-/-}$ BM, megakaryocytes were largely absent and blood vessels appeared more tortuous with increased diameter ($P<0.002$). Expression of TGFβ, platelet factor 4 (PF4) and thrombospondin (TSP)1 was reduced by 70-90% ($P<0.0001$) indicating that these factors are primarily megakaryocyte-derived; VEGF was reduced by 40% ($P<0.01$, Figure 1). Consistent with a modulatory effect of the megakaryocyte-vascular niche on tumour phenotype, in $TPO^{-/-}$ mice, B16 tumour growth was markedly retarded with reduced metastasis to BM and lung. Furthermore, in wild-type mice, BM expression of megakaryocyte-derived VEGF and TGFβ increased during tumour growth while expression of PF4, an autocrine inhibitor of megakaryopoiesis and angiopoiesis decreased, consistent with a role of PF4 in increasing megakaryocyte-vascular niches. Megakaryocyte-conditioned medium (MCM) significantly enhanced proliferation and chemotaxis (P's <0.01) of B16 cells, an effect reduced using MCM from $TSP1^{-/-}$ mice ($P<0.05$). Coculture of megakaryocytes with B16 cells increased megakaryocyte VEGF and TGFβ expression but decreased PF4, consistent with *in vivo* observations, while cocultured

B16 cells displayed increased VEGF and TGFβ and upregulation of adhesion integrins, α4, α5 and α6 (P's <0.05). Moreover, pretreating B16 cells with MCM prior to tail vein injection enhanced tumour engraftment of the lung. Pilot studies to investigate megakaryocyte-tumour interactions in human malignancy showed increased megakaryocytes in trephine biopsies from patients with metastatic disease with and without overt BM metastasis and megakaryocyte clusters with abnormal morphology and localization. Immunohistochemisty of human BM confirmed megakaryocyte localization of VEGF, TGFβ, TSP1, and PF4 and demonstrated megakaryocyte SDF1 staining, suggesting that although megakaryocytes do not produce SDF1 they may 'organize' SDF1 gradients by localizing it to megakaryocyte-vascular niches, thereby influencing homing dynamics of $CXCR4^+$ haematopoietic cells and tumour cells to BM niches. *Conclusions.* Together these findings suggest that megakaryocytes support the integrity of the vascular niche and that homeostasis of the niche may be disrupted in the absence of megakaryocytes. Cellular/molecular cross talk between megakaryocytes and tumour cells at the vascular niche may promote metastasis. Targeting these interactions may be a useful as adjunctive therapy to prevent dissemination of cancer to the BM.

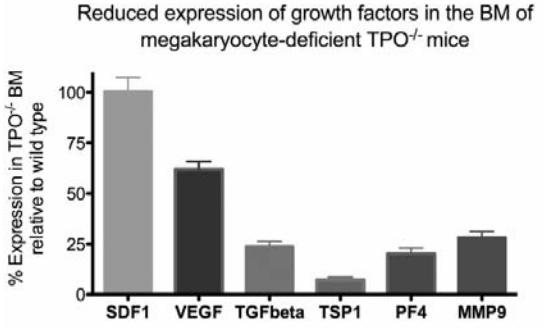

Figure 1.

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THE CDK-INHIBITOR P26INCA1 IS REQUIRED FOR THE MAINTENANCE OF MYELOID LEUKEMIA STEM CELL SELF- RENEWAL

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Acute myeloid leukemia (AML) frequently relapses despite initially effective therapy. Relapse might depend on leukemia stem cells that are not susceptible to standard therapy. A better understanding of biological stem cell regulatory mechanisms might help to design new therapeutic interventions in AML therapy. Stem cell functions in normal hematopoiesis and leukemia relay on the tight regulation of the cell cycle. Recently, we identified p26INCA1 as a novel cell cycle regulator and inhibitor of Cyclin - CDK2 complexes. Loss of Inca1 increased the number of hematopoietic stem cells (HSC) and increased replating efficiency in colony formation assays. Exposing Inca1-deficient mice to cytotoxic stress such as 5- FU exposition induced faster exhaustion of stem cells and early death. These data suggested that Inca could play an important role in HSC expansion and leukemogenesis. To examine the role of Inca1 in leukemogenesis we performed transplantation experiments. We retrovirally transduced wildtype (WT) or Inca1-/- bone marrow cells with AML1-ETO9a and transplanted the retrovirally transduced bone marrow cells into wildtype recipient mice. Five months after transplantation wildtpye recipients developed acute myeloid leukemia. All analysed wildtype recipients showed a leukemic phenotype with an increased white blood cell count and bone marrow infiltration by leukemic blasts. But only one Inca1-deficient mice developed a tumor with myeloid cell infiltration. Further transplantation into secondary recipients demonstrated that AML1-ETO9a induced leukemia in wildtpye mice was transplantable and lethal, while secondary recipients of leukemic Inca1-/- bone marrow did not develop any disease. C-myc induced leukemia developed in both wildtype and Inca1-/- bone marrow. Leukemic bone marrow cells were transplanted into secondary recipients. The frequency of leukemic stem cells was significantly higher in the wildtype bone marrow compared to Inca1 deficient bone marrow. As a consequence only secondary recipients transplanted with leukemic wildtype bone marrow cells died due to leukemia. These findings suggest that the loss of the cell cyle inhibitor p26INCA1 leads to a premature exhaustion of stem cells in the presence of an oncogene. Our results identify an important role of Inca1 in the maintenance of leukemia and potentially the self-renewal of leukemic stem cells.

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THE EMT INDUCER SIP1/ZEB2 IS ESSENTIAL FOR DEFINITIVE EMBRYONIC HEMATOPOIESIS

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Mature blood cells are generated from a small number of hematopoietic stem cells (HSCs) that have enormous regenerative capacity throughout life. The HSC pool size is regulated by a finely controlled self-renewal and differentiation program orchestrated by a number of growth factors and transcription factors. SIP1/ZEB2 is a member of the ZEB family of transcriptional repressors that have been demonstrated to be involved in epithelial to mesenchymal transitions (EMT), processes that direct morphogenesis during development in multicellular organisms. Recently, it was postulated that EMT modulators play key roles in stem cell formation and function, although solid *in vivo* proof of this hypothesis is lacking. Initially we have demonstrated that the EMT inducer SIP1 is expressed in embryonic HSCs, budding out from the hemogenic endothelium in aorta-gonad-mesonephron (AGM) region at embryonic day (E)10,5 and is highly enriched in FACS sorted adult HSCs from wild type mouse bone marrow. Through the use of novel gain and loss of function mouse models, we further investigated the role of SIP1 in HSC formation and function. Conditional loss of SIP1 resulted in embryonic lethality by E12.5 mainly due to cephalic bleedings. This phenotype is very reminiscent of the ubiquitous loss of the hematopoietic transcriptional regulators AML/RUNX1, FLI-1 and GATA-3 that all show identical abnormalities and which are published to be essential for HSC formation. By whole-mount immunostaining and FACS analysis, we have demonstrated that SIP1 is not essential for the formation of HSCs from the AGM region or their migration to the fetal liver. However, SIP1 null hematopoietic progenitors isolated from different hematopoietic organs are unable to differentiate *in vitro* and do not form any hematopoietic colonies. In addition, we observed increased apoptosis and mis-localization of the SIP1 deficient hematopoietic progenitors in the fetal livers. These initial findings suggest key roles for SIP1 in hematopoietic lineage differentiation and stem cell survival. Through the use of these novel mouse models in combination with cell based assays and detailed molecular analysis we will further determine the specific functions and identify hematopoietic targets of the EMT inducer SIP1 in embryonic and adult hematopoiesis and its relevance in leukemia development and progression.

Experimental stem cell transplantation

1160

VASCULAR PROGENITOR CELLS CAN INSTRUCT DEVELOPMENTAL FATE DECISION OF CD146+ MESENCHYMAL STROMAL/STEM CELLS IN VIVO

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Background. Multilineage differentiation potential of mesenchymal stromal/stem cells (MSCs) made them attractive candidates for tissue regeneration purposes. Guiding the differentiation of MSCs towards single lineages would facilitate their application for targeted therapies *in vivo*. MSCs in multiple human organs show a CD146+ perivascular phenotype.[Cell Stem Cell 3:301; 2008] Subendothelial localized self-renewing bone marrow (BM)-derived skeletal stem cells (SSCs) display similar characteristics. These osteoprogenitors are capable of establishing the entire bone and marrow organ including the hematopoietic microenvironment. [Cell 131:324; 2007] We observed that CD146+ MSCs can adapt pericyte phenotype and function to support. vascular regeneration *in vivo* [Blood 113:6716; 2009] in addition to their mesodermal lineage differentiation potential. We hypothesized that environmental conditioning by ECFCs plays a role during the developmental fate decision of MSCs *in vivo*. *Methods.* CD146+ SSCs/MSCs as well as CD146+ endothelial colony forming progenitor cells (ECFCs) were isolated from adult BM, umbilical cord blood (UCB) and perivascular cord tissue. Proliferation potential and clonogenicity were monitored. Phenotype was analyzed by flow cytometry and immune cytochemistry. Cell function was studied in differentiation assays and during vascular network assembly *in vitro*. Models for *in vivo* human vessel as compared to bone formation were established in immune-deficient mice. Non-invasive imaging was performed using computed tomography (CT), magnetic resonance (MRI) and fluorescence imaging. Immune histochemistry was applied for morphologic studies in the time course of organogenesis. *Results.* Baseline analysis confirmed MSC and ECFC purity, immune phenotype and sustained proliferation potential. Progenitor cell hierarchy within ECFC cultures was preserved after oligoclonal large-scale expansion. We could show that human BM-derived CD146+ MSCs are capable of forming bone and the marrow organ *in vivo*. Co-transplanted human ECFCs could instruct the MSCs to differentiate either into pericytes or chondrocytes and osteoblasts, depending on the applied MSC/ECFC cell ratio. Non-invasive imaging and histological staining revealed that ectopic organogenesis had already started after 2-4 weeks and was stable during the observation period of 20 weeks. Non-BM-derived CD146+ populations, although phenotypically resembling SSCs, invariably lacked the capacity to build bone and marrow environment *in vivo*. *Conclusion.* This demonstrates that human ECFCs can instruct MSCs and induce developmental fate decisions early in the time course of organ regeneration after transplantation. This model is a promising tool to study the therapeutic applicability, modulation and risk profile of ECFC/MSC-based transplantation strategies.

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DIFFERENCES IN ALLOREACTIVE T CELL MIGRATION IN MHC MATCHED VERSUS MHC MISMATCHED HCT ARE CAUSED BY WAVES OF T CELL EXPANSION

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Background. Acute graft-versus-host disease (aGvHD) can be characterized by an initiation phase (activation and proliferation of alloreactive T cells) and an effector phase (infiltration of target tissues). Yet, it remains important to better understand aGHVD pathogenesis to pinpoint critical time points for improved diagnostic and therapeutic interventions. *Aim.* To analyze the course of T cell activation, proliferation and homing in a clinical relevant fully MHC-matched transplantation mouse model with mismatches in minor histocompatibility antigens. *Methods.* Luciferase-labeled C57Bl/6 T cells were transplanted into MHC mismatched Balb/c or MHC matched Balb/b recipients. Donor T cell migration was visualized by *in vivo* bioluminescence imaging (BLI). Activation and proliferation were analyzed using multiparameter flow cytometry. GVHD scoring was performed by histopathology. *Results.* By closely monitoring light emitting donor T cells for 30 days after allogeneic hematopoietic cell transplantation (allo-HCT) we observed that they highly proliferated exclusively in secondary lymphoid organs (initiation phase) in both models until day+3. Between day+3 and day+5 T cells migrated via the peripheral blood into target organs (effector phase). Both models showed similar cell distribution patterns but markedly differed in absolute T cell expansion and gastrointestinal tract, liver and skin infiltration. When comparing the BLI results in the MHC mismatched model a continuous strong signal increase could be measured from day+3 to day+6 (55-fold increase by day+6 as compared to day+3). T cell expansion peaked between day+6 and day+8 according to highest photon emission rates. In contrast, in the MHC matched model whole body BLI signals increased constantly but slower (10-fold increase by day+6 as compared to day+3) as well as for single target organs until day+30. BLI signal increase resembled an undulating curve with plateaus at certain time points (day+11, day+15, day+21) rather than a continuous signal accumulation. Clinical GVHD symptoms appeared after day+23. Syngeneic transplanted mice demonstrated a steady signal increase due to T cell expansion and not an undulating course of amplifying proliferation loops. Daily flow cytometric measurements of alloreactive T cell numbers in the peripheral blood confirmed these *Results.* CD4+ donor T cells left the secondary lymphoid organs shortly before CD8+ donor T cells in both models but in the MHC mismatched model absolute cell numbers increased faster (peak on day+6) compared to the MHC matched situation (peak on day+11). Additionally, alloreactive peripheral blood CD8+ T cells upregulated certain tissue specific homing receptors (e.g. $\alpha 4\beta 7$, CXCR3, P-selectin ligand) and activation markers (e.g. CD44, CD25). These results correlated with subsequent histopathological GVHD-scorings of transplanted mice. *Conclusion.* Our results in this clinical relevant mouse model provide evidence for progressive waves of T cell expansion and migration rather than a continuous increase in alloreactive T cell numbers. As alloractive T cells expand at early time points, before aGVHD becomes apparent, our results offer an improved basis for optimized diagnostics and therapeutic interventions.

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FUNCTIONAL IMMUNE RECOVERY AFTER ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANTATION: NASCENT DONOR T CELLS ARE SUPERIOR TO MATURE GRAFT T CELLS IN THEIR REACTIVITY AGAINST CYTOMEGALOVIRUS

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Infections due to impaired immune recovery are a major cause of morbity and mortality after allogeneic hematopoietic cell transplantation (HCT). Donor (do) T cells (TC) in the graft are thought to provide protective immunity, as TC-depleted grafts are associated with increased rates of viral infections post HCT (pTX). However, TC-containing grafts require stronger pharmacologic immunosuppression, and doTC target lymphoid organs with subsequent immune dysfunction. Using an MHC-matched, minor antigen (miAg) mismatched GVHD model, we compared immune response against murine cytomegalovirus (MCMV) in recipients of purified hematopoietic stem cells (HSC; cKit+Sca1+Thy1.1loLin-) *vs.* HSC supplemented with TC. Anti-viral reactivity was assessed for (1) transferred doTC from uninfected and pre-immunized donors; (2) HSC-derived doTC, and (3) residual host TC. Lethally irradiated BALB.B mice were infused with C57BL/6 (B6) HSC or HSC+TC (TC from CD45 congenic B6). 2 or 8 weeks (w) pTX hosts were infected with sublethal doses of MCMV. 2w later lymphoid organs were harvested to assess anti-MCMV reactivity of CD8+ TC using M45 tetramers. Splenocytes were separated into doTC-, doHSC, and host-derived populations, to compare their IFNγ production in an MCMV ELISPOT assay. Mice infected at 2w pTX (modeling early CMV reactivation) and given TC-replete grafts mounted significantly lower tetramer responses (attributable to doTC-derived cells), than control congenic recipients, or recipients of HSC alone. In the latter group tetramer-reactive cells originated from the residual host. ELISPOT analysis confirmed that best responses were mounted by doTC transplanted into control congenic mice, followed by host-type TC. Pre-sensitization of TC donors did not improve the low response of doTC transplanted into miAG-mismatched hosts. However, when HSC were supplemented with naïve (CD62L+CD44-) or memory (CD62L±CD44+)

CD8+ TC from immunized donors, there were no signs of GVHD and recipients of memory, but not naïve CD8 TC mounted strong responses in both assays. At 8w pTX recipients of pure HSC were mixed donor/host TC chimeras. Mice given TC-replete grafts were full donor chimeras with the majority of TC originating from the transferred doTC. When mice were infected 8w pTX anti-MCMV reactivity of $CD8^+$ TC in tetramer and ELISPOT assays were strikingly superior in recipients of pure HSC, as compared to weak responses seen with TC-replete grafts. In recipients of pure HSC donor and host populations demonstrated strong reactivity, while mice given TC-replete grafts not only had low responses of the doTC-, but also of the doHSC-derived TC (Figure 1). Thus, our key findings are 1.) Mature doTC do not maintain their full anti-viral potential when transplanted into miAg-disparate mice, even when obtained from pre-immunized donors. 2.) Residual host cells contribute substantially to protective immunity, but are eradicated by conventional TC-replete grafts. 3.) HSC-derived TC arising in a healthy host are superior to those that develop and undergo selection in a GVHD-affected lymphoid system. Our results are of critical importance as they question the conventional assumption that doTC are required for optimal protection. Rather, our studies suggest that long-term immune function will greatly benefit from rigorously TC-depleted grafts and a GVHD-free lymphatic environment.

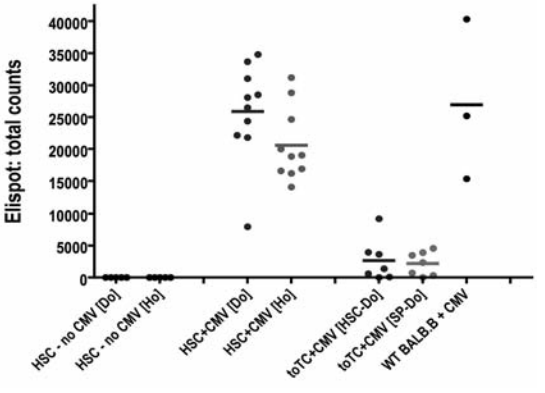

Figure 1. Total counts (Elispot) of CMV reactive CD8 TC.

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CD11B+CD11C+ DENDRITIC CELLS INTERACT WITH ALLOREACTIVE T CELLS IN THE INTESTINAL MUCOSA IN ACUTE GRAFT-VERSUS-HOST DISEASE

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Background. Recipient dendritic cell - donor T cell interactions in secondary lymphoid organs are essential to initiate acute graft-versus-host disease (aGVHD). Yet, to which extend mucosal dendritic cells (DCs) contribute to augment, maintain or even down-modulate aGVHD in peripheral tissues remains elusive. *Aim.* To investigate whether DCs in the intestinal mucosa are required to perpetuate aGVHD. *Methods.* To address this question we used a murine allogeneic hematopoietic cell transplantation (allo-HCT) model [luciferase+ FVB/N (H-2q) or C57Bl/6 (H-2b) splenocytes (CD90.1)] plus wild type bone marrow into myeloablative conditioned allogeneic Balb/c recipients (H-2d, CD90.2, 8Gy).To visualize cell migration we employed non-invasive bioluminescence imaging. Phenotyping studies were performed by using multiparameter flow cytometry, and cell interaction was analyzed by fluorescence/confocal microscopy. *Results.* Analysis of the intestinal mucosa revealed high numbers of DC - donor T cell contacts during the aGVHD effector phase. Persisting host APCs (I-Ad) co-localized with both, $CD4^+$ $CD90.1^+$ and $CD8^+$ $CD90.1^+$ donor T cell subtypes. Although myeloablative conditioning augmented the numbers of infiltrating alloreactive T cell in the intestinal tract, we observed no reduction in DC - donor T cell contacts in non-conditioned recipients when compared to conditioned Balb/c Rag-/-common-γ-chain-/- allo-HCT recipients. Next we investigated which peripheral mucosal DC subtypes engaged with donor T cells. DC subsets shifted from migratory $CD103^+$ CD11blo $CD11c^+$ DCs, observed during steady state conditions, to a dominating $CD103^-$ CD11bhi $CD11c^+$ DC population during the aGVHD effector phase. Confocal microscopy confirmed interactions between this DC subset and donor T cells and at sites of contact revealed engagement of costimulatory molecules such as CD80. *Conclusion.* Our data provide evidence for a likely underappreciated role of mucosal DCs during later stages of aGVHD. This suggests that disruption of mucosal DC - donor T cell interactions may provide an attractive therapeutic target in patients suffering from persisting therapy refractory GVHD.

AB and SS contributed equally to this work.

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UMBILICAL CORD BLOOD REGULATORY T CELLS: FUNCTIONAL ANALYSIS AND GENE PROFILING

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Background. Cord blood (CB) transplant is characterized by a reduced incidence of graft-versus-host disease (GVHD). The possible role played by CB regulatory T cells (Tregs) for the suppression of the allogeneic T-cell response is now under investigation. *Aims.* Aim of this study was to analyze and compare the functional properties and gene expression profile of Tregs expanded from CB units with those expanded from the peripheral blood (PB) of normal donors. *Methods.* Tregs were purified from mononuclear cells obtained from 23 CB units and from the PB of 13 adult normal donors using the Treg isolation kit (Miltenyi Biotec) and expanded for 6 days with anti-CD3, anti-CD28 and IL-2. Immunophenotypic analyses were performed before and after expansion. In order to measure the suppressor activity of expanded Tregs, mixed lymphocyte reaction (MLR) cultures were performed with autologous effector T cells stimulated with allogeneic dendritic cells (DC) in the presence or absence of Tregs. The IL-10 production capacity was tested using an ELISA assay. Gene expression profile experiments were performed using the HGU133 Plus 2.0 arrays (Affymetrix); statistical and functional annotation analysis were carried out using the dChip and the DAVID software. *Results.* CB and PB Tregs presented similar immunophenotypic appearances; after expansion, a comparable expression of surface CD4, CD25, CD62L, CCR5 and CD45RO, and of cytoplasmic CTLA-4 and Foxp3 was observed; in addition, they both were negative for the CD45RA antigen, indicating the loss of their naïve features. On the contrary, Tregs obtained from CB (n=23) presented a significantly higher expansion capacity compared to those obtained from PB (n=13): mean fold increase (range), CB 10.3 (1.6-24), PB 3.9 (1.5-10), p 0.003. CB expanded Tregs (n=6) exerted a potent suppressive function on MLR that resulted slightly but not significantly inferior to that exerted by PB expanded Tregs (n=5): mean fold suppression (range), CB 7.8 (2.5-15.1), PB 14.3 (1.5-23.7), p 0.14. Tregs expanded from CB (n=5) and PB (n=3) presented a high and comparable *in vitro* IL-10 production capacity: mean pg/mL (range), CB 325.6 (320-334), PB 341.3 (319-382). Gene expression profile analysis revealed 481 probesets differentially expressed between CB (n=4) and PB (n=6) expanded Tregs; functional analysis showed in CB Tregs a significant enrichment of genes involved in cell proliferation, chromatin modification and regulation of gene expression, thus possibly providing an advantage in cell expansion. In contrast, PB expanded Tregs showed an overexpression of genes involved in the adaptive immune response, including several cytokines and/or their receptors. Finally, when looking at the Foxp3 gene expression levels, no differences were observed between the two populations. *Summary/Conclusions.* These results demonstrate that Tregs contained in CB units retain an expansion potential superior to Tregs isolated from the PB of normal donors. The maintenance of the modulatory properties after expansion is confirmed by the expression of the Foxp3 gene and protein, and by the production of IL-10. These data offer further insights into the understanding of the biology of CB transplantation, indicating a possible role played by CB Tregs in the suppression of the allogeneic T-cell response.

Prognostic markers in acute myeloid leukemia

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FURTHER STUDIES IN CEBPA DOUBLE MUTATIONS AS REGARDS TO FAVOURABLE PROGNOSTIC IMPACT, RELATION WITH OTHER GENE MUTATIONS AND IMPROVED GENE EXPRESSION SIGNATURE IN A LARGE COHORT OF AML

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Introduction. CEBPA, a gene encoding a transcription factor that is essential for myeloid development, is mutated in 8-10 percent of human cytogenetically normal acute myeloid leukemia (CN-AML). CEBPA mutant AML can be subdivided into two categories, i.e. cases with mutations of both alleles (CEBPAdouble-mut) or with single mutations (CEBPAsingle-mut). We previously reported that patients with a CEBPAdouble-mut represent a distinct AML subtype with a unique gene expression profile (GEP) and a more favourable outcome, this in contrast to CEBPAsingle-mut AML. *Aims.* In this study we examined the distribution of CEBPAsingle-mut and CEBPAdouble-mut in a large *de novo* adult CN-AML cohort (n=1175) and studied the impact on survival. Furthermore, we investigated whether a previously defined CEBPAdouble-mut GEP-signature could be improved using continuous selection procedures, i.e., Lasso regulation. *Methods.* AML patients (age ≤60) were included from the Dutch-Belgian-Swiss HOVON-SAKK and German-Austrian AMLSG and SHG studies. For CEBPA mutational screening, denaturing high performance liquid chromatography and nucleotide sequencing was performed. Gene expression profiles were derived using Affymetrix (Santa Clara, CA, USA) HGU133Plus 2.0 GeneChips. Unsupervised analyses were performed using hierarchical clustering based on Pearsons correlation coefficients. Prediction analyses were done using Lasso regulation, as it takes into account the correlation structure between the genes and selects genes in a continuous way such that their combinatorial effect is stronger than discrete selection of genes, e.g. Prediction Analyses of Microarrays (PAM). *Results.* Among the 1175 CN-AML cases we identified 88 $CEBPA^{double\text{-}mut}$ and 57 $CEBPA^{single\text{-}mut}$ patients. A significant favourable outcome was observed for $CEBPA^{double\text{-}mut}$ AML as compared to $CEBPA^{wt}$ ($P<0.001$, HR=0.3). In this analysis $CEBPA^{single\text{-}mut}$ group showed a favourable outcome as well ($P=0.03$, HR=0.6). Importantly, the latter group showed a significantly higher incidence of concurrent mutations in NPM1 and FLT3 ($FLT3^{ITD}$) than the $CEBPA^{double\text{-}mut}$ group ($P=7\times10^{-8}$ and $P=1\times10^{-4}$ respectively). These concurrent mutations are indicative for the clinical outcome within the $CEBPA^{single\text{-}mut}$ group. A favourable response was observed for $CEBPA^{single\text{-}mut}$/$NPM1^{mut}$/$FLT3^{wt}$ AML as compared to $CEBPA^{wt}$ ($P=0.03$), whereas $CEBPA^{single\text{-}mut}$/$NPM1^{wt}$/$FLT3^{ITD}$ AMLs showed an unfavourable trend with regard to therapy. These data reflect the predictive value of NPM1 mutations and $FLT3^{ITD}$ in CN-AML. Unsupervised analysis of GEP data (n=674) showed that $CEBPA^{double\text{-}mut}$ (n=42) and not $CEBPA^{single\text{-}mut}$ (n=18) AML clustered tightly together. Moreover, by using Lasso we derived a 25 GEP-signature that outperformed the previous $CEBPA^{double\text{-}mut}$ GEP-signature derived with PAM. This has the additional advantage that it can correctly classify AML cases with hypermethylation of the proximal promoter region of CEBPA. This increased the classification of $CEBPA^{double\text{-}mut}$ to 100% sensitivity and specificity in both training as well as validation set. *Conclusions.* We showed that $CEBPA^{double\text{-}mut}$ AML is a distinct AML subtype in contrast to $CEBPA^{single\text{-}mut}$ AML, in terms of molecular characteristics, clinical outcome and GEP. The $CEBPA^{single\text{-}mut}$ group has a higher incidence of concurrent mutations, in which $FLT3^{ITD}$ and NPM1 mutations are indicative for the prognostic relevance. The $CEBPA^{double\text{-}mut}$ group is a homogeneous group which has a favourable outcome and can be classified with excellent accuracy by GEP.

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PROGNOSTIC IMPORTANCE OF HUMAN MIXED LINEAGE LEUKEMIA 5 (MLL5) EXPRESSION LEVELS IN ACUTE MYELOID LEUKEMIA

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Background. The human Mixed-Lineage-Leukemia-5 (MLL5) gene is located on chromosome 7q22 in a genomic region frequently deleted in patients with myeloid malignancies. Monosomy 7 or deletions of the long arm of chromosome 7 are asscociated with poor outcome in AML and MDS. Recently, functional analysis revealed that MLL5 is involved in terminal myeloid differentiation and in regulating proliferation and functional integrity of hematopoietic stem/progenitor cells. In homozygous loss-of-function Mll5 mice, a combined maturation defect of the myeloid and erythroid lineage has been described. *Aims.* In the present study we investigated the prognostic impact of MLL5 expression levels in 509 AML patients. *Methods.* All patients were uniformly treated according to AML SHG 0295 or AML SHG 0199 treatment protocols. RNA was extracted from diagnostic bone marrow or peripheral blood cells, reversely transcribed, and amplified by quantitative RT-PCR. RT-PCR was carried out on a StepOne Plus real-time PCR system using ABL as an endogenous control. Statistical analyses were performed using SPSS. Patients were divided into quartiles by MLL5 expression values. *Results.* While patients with low MLL5 transcript levels in quartiles 2, 3 or 4 did not show a significant difference in outcome when compared with each other in univariate analysis, the 127 patients with high MLL5 expression (quartile 1) had a higher CR rate (83% v 74%; P=.02), a longer RFS (P=.009) and a longer OS (P=.001) compared with the 382 patients with lower MLL5 expression. No correlation between MLL5 expression and cytogenetic aberrations was found. When analyzing the prognostic impact of MLL5 expression in cytogenetically normal AML (CN-AML, n=268), high MLL5 expressers had a longer RFS (P=.03) and longer OS (P=.007). In multivariate analysis for RFS and OS, MLL5 expression status was an independent favorable prognostic factor (RFS: HR 0.71, 95% CI 0.5 to 0.98, P = .041; OS: HR 0.65, 95% CI 0.48 to 0.88, P=.005) in the total cohort of 509 patients when considered together with age, karyotype, WBC count, platelet count, and type of AML (*de novo vs.* secondary). To test the prognostic value of MLL5 expression in the context of other prognostic molecular markers in CN-AML, multivariate analysis for OS was performed also considering the NPM1/FLT3 and the CEBPA mutation status, the WT1 SNP rs 16754 status, the WT1, MN1, BAALC, and the ERG expression status, age, WBC count, and platelet count. Strikingly, MLL5 remained an independent favorable prognostic marker in the context of this well characterized cohort (HR 0.58, 95% CI 0.36 to 0.94, P=.028). *Conclusion.* We identified MLL5 transcript levels as an independent favorable risk marker in AML, enabling the identification of a significant proportion of patients with favorable prognosis that are neither identified by mutational nor cytogenetic analysis.

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SINGLE CELL NETWORK PROFILING (SCNP) OFFERS A NOVEL APPROACH TO IDENTIFY AT DIAGNOSIS AML CHEMOTHERAPY RESISTANT CELL PHENOTYPES

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Background. Despite improvements in AML response to initial chemotherapy the risk of relapse, especially in older patients, remains high. While chemotherapy regimens can eliminate the majority of the AML blasts, the remaining cells, ultimately responsible for disease relapse, have unique characteristics that render them resistant to the chemotherapy. Current tools to predict disease relapse at diagnosis are inexact. In fact, while many genomic (e.g. gene expression arrays) or proteomic (e.g. reverse phase protein arrays) technologies are capable of revealing the biology of "bulk" AML, biologic tools that detect small chemo-resistant cell populations in AML samples are needed. Single cell network profiling (SCNP) is a biologic tool using multiparameter flow cytometry that allows a comprehensive functional assessment of intracellular signaling pathways in heterogeneous tissues at the single cell level. *Aims.* Our aims were to use SCNP to determine whether: a) in longitudinally collected AML samples, cell surface phenotypes and/or intracellular signaling profiles dominant at relapse could be identified in subpopulations of cells present at diagnosis and b) whether the presence of (rare) blasts with these intracellular signaling profiles could predict for disease relapse in an independent set of AML diagnostic samples. *Methods.* Three paired diagnostic and relapse AML bone marrow samples were examined using SCNP after sample incubation with cytokines, growth factors, chemotherapeutic agents, and other modulators. The use of fluorochrome-conjugated antibodies that recognized leukemic blasts and intracellular phospho-epitopes allowed signaling to be measured in specific cell types at the single cell level. In addition, drug transporters and surface receptor levels were also measured. *Results.* Analysis of the myeloblast subpopulations as defined by surface markers revealed heterogeneity between samples (both at diagnosis and relapse) which was not informative in term of relapse risk. By contrast, the intrapatient characterization of intracellular signaling profiles between relapsed and diagnostic samples revealed in all the three relapsed samples the presence of a subpopulation of leukemic cells characterized by simultaneous phosphorylation of Akt and S6 in response to SCF (SCF:p-Akt/p-S6). This functionally defined leukemic subset, although dominant in the relapse samples, was detectable at a much lower frequency in the diagnostic samples. We hypothesized that the presence of this SCF:p-Akt/p-S6 subpopulation at diagnosis could be predictive for early disease relapse and applied the SCF:p-Akt/p-S6 gate to an independent SCNP data set containing 52 diagnostic AML samples from patients who achieved complete remission (CR) after standard induction therapy. Seven of those patients showed a detectable SCF:p-Akt/p-S6 subpopulation in their diagnostic samples and six of the latter patients experienced disease relapse within 2 years. Of note, the presence of a SCF:p-Akt/p-S6 subpopulation was shown to be independent from the blasts c-Kit (SCF receptor) expression levels. In addition, the SCF:p-Akt/p-S6 profile was independent from patient age, AML cytogenetics and Flt-3 mutational status. *Conclusions.* Our study showed that longitudinal SCNP analysis of AML samples could provide unique insights into the nature of AML chemo-resistance allowing for identification of subpopulations of cells present at diagnosis with unique signaling characteristics predictive of higher rates of relapse.

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A STUDY ON THE CLINICAL SIGNIFICANCE OF THE WHO AML SUBTYPE INV(3)(Q21Q26.2)/T(3;3)(Q21;Q26.2) AND VARIOUS OTHER 3Q CHROMOSOMAL ABNORMALITIES IN 6,819 AML CASES

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Background. Among patients with newly diagnosed acute myeloid leukemia (AML), inv(3)(q21q26.2) or t(3;3)(q21;q26.2) [inv(3)/t(3;3)] occurs in 1-2% of the cases. Although it has been reported that the prognosis of inv(3)/t(3;3) AML is poor and the disease associates with aberrant EVI1 expression, a comprehensive study on this AML subtype is lacking due its low frequency. Furthermore, the clinical significance and molecular basis of the diversity of other 3q rearrangements involving either chromosome 3q26 or 3q21 remain to be elucidated. *Aim and Methods.* We evaluated the frequency and the clinical significance of AML with inv(3)/t(3;3) and various other 3q abnormalities in comparison to non-3q cytogenetically abnormal (CA) AML in 6,819 newly diagnosed adult AML patients treated on Dutch-Belgian-Swiss HOVON/SAKK (n=3,318) and German-Austrian AMLSG protocols (n=3,501). In a subset of cases, we assessed for aberrant EVI1 and MDS1/EVI1 expression using real-time quantitative PCR (qPCR), and we studied the association with common gene mutations. *Results.* Chromosome 3q abnormalities were detected in 4.2% of AML cases (n=288/6,819). Patients ≤ 60 years of age more frequently carried a 3q abnormality (4.5%; 231/5,061) than patients > 60 years (3.2%; 67/1,758). Four different AML subgroups with distinct 3q aberrations were considered: (A) inv(3)/t(3;3) (WHO defined subtype of AML, n=94); (B) other balanced 3q26 translocations, e.g. t(3;12)(q26.2;p13), t(3;21)(q26.2;q22) (n=52); (C) other balanced 3q21 translocations, e.g. t(1;3)(p36;q21), t(3;5)(q21;q31) (n=19); (D) other 3q abnormalities (n=123). Patients with inv(3)/t(3;3) or balanced 3q21 translocations presented with significantly higher white blood cell (WBC) and platelet counts. Monosomy 7 was a common additional aberration, in particular in Group A (66%), but also in Groups B (31%) and D (37%). N-RAS mutations were significantly associated with inv(3)/t(3;3) and balanced 3q26 translocations. All four 3q groups showed poor survival outcomes; within inv(3)/t(3;3) cases, the adverse prognostic impact was strengthened by the presence of monosomy 7. In addition, the prognostic impact of the 3q groups was determined by multivariable analysis considering the variables age, WBC and platelets counts, monosomy 7, monosomal karyotype, complex karyotype, and type of AML. Inv(3)/t(3;3) (Group A) independently predicted low CR rate (OR; 0.29, P<.0001), adverse relapse-free survival (HR, 2.0, P<.0001), event-free survival (HR, 2.0, P<.0001), and overall survival (HR, 1.4, P=.006). EVI1 was highly expressed in Groups A and B, but the disproportionate EVI1 *vs.* MDS1/EVI1 overexpression was characteristic of inv(3)/t(3;3) AML. Interestingly, disproportionate EVI1 *vs.* MDS1/EVI1 overexpression was also found in a small subset of Group D 3q aberrant AML. Most of these AML carried del(3)(q26), add(3)(q26), del(3)(q21) or add(3)(q21) pointing to the presence of cryptic inv(3)/t(3;3) lesions leading to altered EVI1/MDS1/EVI1 expression. *Summary/Conclusion.* AML with inv(3)/t(3;3) represents a distinctive subgroup with poor prognosis, which discriminates from most other AML with 3q aberrations. Adverse prognostic impact was further enhanced by additional monosomy 7. EVI1/MDS1/EVI1 qPCR may allow for the identification of cryptic inv(3)/t(3;3) AML.

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RUNX1 MUTATIONS FORM A DISTINCT MOLECULAR SUBGROUP IN ACUTE MYELOID LEUKEMIA: RESULTS OF THE AML STUDY GROUP (AMLSG)

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Background. The highly conserved runt-related transcription factor 1 gene (*RUNX1*) is required for definitive hematopoiesis; functional dysregulation of *RUNX1* has been shown to be involved in leukemogenesis. In acute myeloid leukemia (AML) *RUNX1* is implicated by chromosomal translocations [e.g. *RUNX1-RUNX1T1* in t(8;21)], point mutations or amplifications. In addition, *RUNX1* germline mutations that can be found in patients with familial platelet disorder predispose to the development of myeloid leukemia. Recent data suggest that *RUNX1* mutations are related to distinct cytogenetic subgroups such as trisomy 13, trisomy 21, loss of chromosome 7q, and trisomy 8. *Aims.* To characterize the biological and clinical features of *RUNX1* mutations in a large cohort of younger (16 to 60 years of age) adult AML patients (pts) who were entered on two consecutive AMLSG treatment protocols (AML HD98A, AMLSG 07-04). *Methods. RUNX1* mutation screening was performed in 977 AML-pts (*de novo* AML, n=904; s-AML, n=27; t-AML, n=38) using a DNA-based PCR assay for amplification of exons 1 to 8 followed by direct sequencing. For a subset of cases (n=269) gene expression profiling (GEP) was performed using 40k cDNA microarrays. *Results.* The incidence of *RUNX1* mutations was 5.5% in the entire cohort (54 of 977 pts); mutations clustered in exon 3 (10) and exon 8 (21), but were also located in other regions of the gene. With regard to cytogenetic subgroups, *RUNX1* mutations mainly occurred in the intermediate risk group [7,1%; 47/657] predominantly in normal karyotype AML (CN-AML; 32/546); in the high risk group the frequency was lower [4,5%; 5/106] whereas in the low risk group no *RUNX1* mutations were identified [0/146]. With respect to pts characteristics such as age, WBC, LDH and platelet count there were no differences between the *RUNX1* mutated (*RUNX1*^{mut}) and *RUNX1* wildtype (*RUNX1*^{wt}) group. *RUNX1*^{mut} were more frequent in s-AML (4/27) compared to t-AML (3/38) and *de novo* AML (46/904) (P=0.05). Correlations with molecular markers (*NPM1*, *FLT3*-ITD, *FLT3*-TKD, *MLL*-PTD and in addition for CN-AML *WT1*, *CEBPA*, *NRAS*) revealed a significant association of *RUNX1*^{mut} with *MLL*-PTD (P=0.0005) and an inverse correlation with *NPM1* mutations (P<0.0001). Furthermore, *RUNX1*^{mut} showed a characteristic gene expression pattern characterized by the enrichment of genes belonging to pathways associated with TNF signalling and apoptosis. These findings suggest a distinct biology for *RUNX1*^{mut} AML that also seems to translate clinically as *RUNX1*^{mut} pts had a higher rate of resistant disease (RD) with 30% refractory cases compared to 16% for *RUNX1*^{wt} pts (P=0.02). In accordance, *RUNX1*^{mut} cases showed a significantly lower complete remission (CR) rate (61% *vs.* 75%, P=0.04). In univariable analysis, event-free survival (EFS; P=0.00003), relapse-free survival (RFS; P=0.007), and overall survival (OS; P=0.02) were significantly inferior in *RUNX1*^{mut} pts; in multivariable analysis, *RUNX1*^{mut} was an independent prognostic marker for lower EFS. *Conclusions.* In younger adult AML-pts, *RUNX1* mutations mainly occur in the intermediate risk group, in particular in CN-AML. Correlations of *RUNX1*^{mut} with clinical, genetic and gene expression data suggest that this gene mutation confers a distinct biological impact that is associated with inferior outcome in younger AML-pts.

Publication only

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ROLE OF CYTOCHROME P1A1 AND GLUTATHIONE S TRANSFERASE GENE POLYMORPHISMS IN THE PATHOGENESIS OF EGYPTIAN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. Xenobiotic-metabolizing enzymes constitute an important line of defense against a variety of carcinogens. Many are polymorphic, constituting the basis for the wide inter-individual variation in metabolic capacity and possibly a source of variation in the susceptibility to chemical-induced carcinogenesis. *Aims.* The aim of this study was to determine the existence of any association between the genetic polymorphisms of P450 1A1 (CYP1A1) & glutathione S-transferase M1, T1&P1 (GSTM1, T1&P1), and altered risk for pediatric ALL. *Methods.* A total of 92 patients and 314 controls were genotyped by means of PCR and PCR-RFLP-based assays. Mutated alleles comprising CYP1A1*2A,*2B,*4, GSTM1*Null, GSTT1*Null, GSTP1Ile105Val and Ala114Val, were analyzed along with the wild-type alleles. *Results.* The frequency of the GSTP1 114Ala/Val (2293CT) heterozygous genotype was 16(17.9 %) among patients compared to 27 (8.3 %) among controls; the difference was found to be statistically significant (P=<0.001, O.R=2.494 & 95% C.I 0=1.272-4.890). The frequency of the GSTP1 114Val/Val (2293TT) homozygous genotype was 6 (6.7 %) among patients compared to 1 (0.3 %) among controls; the difference was found to be statistically insignificant (P=<0.001, O.R=25.254 & 95% C.I 0=2.99-213.294). this results pointed to 2.5 folds and 25 fold increased risk of ALL for both heterozygous and homozygous respectively. although a near significant increase in GSTP1 Ile105Val was also found in ALL group (P = 0.054). Our results showed no significant association between the CYP1A1 *2A, *2B and*4 genotypes and the risk of childhood ALL. Also the combined genotype of the three polymorphisms did not affect the ALL risk. Also no statistically significant difference for GSTM1 null or deletion genotype was encountered between patients and controls (P=0.553). *Summary and Conclusion.* The results suggest an association of impaired Xenobiotic-metabolizing enzymes specially GSTP1 activity with an increased risk for pediatric ALL, possibly by a decrease in the metabolic detoxification of chemical carcinogens. Further wide scale studies will be needed to confirm such effect on ALL pathogenesis.

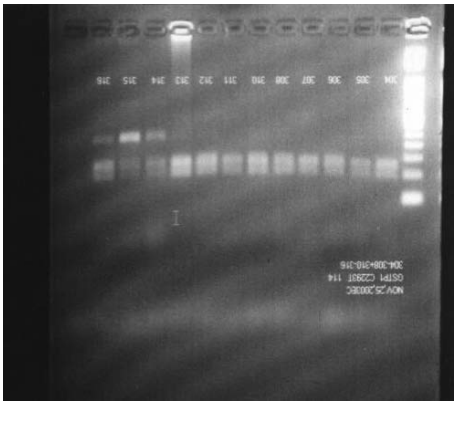

Figure 1. GSTP1 Ala114Val polymorphism.

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A NEW ENTITY OF T LINEAGE ACUTE LYMPHOBLASTIC LEUKEMIA (T-ALL) RESPONDING TO TYROSINE KINASE INHIBITOR (IMATINIB MESYLATE): COMPLETE CHARACTERIZATION OF A PEDIATRIC CASE WITH RESISTANT DISEASE

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Background. T cell acute lymphoblastic leukemia (T-ALL) accounts for 15% of pediatric ALL cases. Prognosis has improved in recent years but some cases suffer from relapse/resistant diseases, which are mostly fatal. Application of new methodologies have shown that several genes with tyrosine kinase activity are involved in the leukemogenic process, co-operating with pro-survival molecules. *Aim.* To characterize a case of pediatric T-ALL who showed a clinical response to Imatinib Mesylate (IM). *Methods.* We performed immunophenotypic, conventional karyotype, FISH, RT-PCR, sequencing and western-blot analyses, detection of minimal residual disease (MRD) using TcR rearrangements, SNPs arrays using GeneChip Operating Software (GCOS) and Genotyping Console GTC 3.0.1. *Results.* A 4-year-old black boy from Mauritius with T-ALL was firstly treated with a FRALLE-2000 and further with AIEOP-BFM-2000 protocols. During maintenance, he showed a bone marrow isolated relapse with FAB L2 and myeloid features and aberrant expression of CD10, CD13 and CD117/cKit antigens. Karyotypic analyses showed a 47,XY,+8,del(11)(q13q23). FISH, molecular screening and SNPs analyses excluded the MLL gene involvement. SIL/TAL1 del(1), and NUP214/Abl episomes were not detected. The child was treated with two courses of Idarubicin-CytosineArabinoside, but always recovered with circulating blasts. Based on cKit expression and trisomy of chromosome 8, we designed a therapy with IM (300 mg/mq/daily) followed by a course with Etoposide and Cyclophosphamide. After few days of IM administration, we observed a rapid reduction of peripheral blasts count and splenomegaly. He achieved a partial remission, which persisted for 6 months. Afterwards he suffered from a relapse and died for progression disease. Sequencing analyses of cKit gene did not show any alteration. We retrospectively performed the detection of MRD, confirming that the clone of relapse was the same as diagnosis, because of ots persistence during first line therapy. By SNPs, we found two 1.7 Mb deletions: one in band 9p21.3 (CDKN2 genes) and the other in band 16q22.1 (NFATC3 gene). FGFR2 , cKit, FGFR3, FGFR4, PDGFRB and ABL1 genes, encoding putative IM targets, did not show copy number abnormalities or loss of heterozigosity. Conversely we observed a somatic loss of 0.6 Mb at 4q12 includes part of the 5'region of the PDGFRA gene encoding a tyrosine kinase receptor. A del(4)(q12q12) of about 800 kb generating the fusion between the 5'part of the FIP1L1 gene and the 3'part of the PDGFRA has been described as the molecular abnormality responsible for the development of idiopathic hypereosinophilia which usually responds to IM. We here detected a fusion transcript, strongly suggesting a biological basis of response to IM. We also found by western blot analyses an up-regulation of AKT pathway associated with a downregulation of PTEN, a potential marker of disease and a novel target of treatment. *Conclusions.* We pointed out on a potential new entity of T-ALL subtype which could benefit from treatment with tyrosine kinase inhibitors. We suggest to take into account the role of cKit expression, trisomy of chromosome 8, and mainly PDGRA-FIP1L1 fusion transcript as molecular alterations for a targeted therapy as well as AB1 fusion genes. Further studies are needed.

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E2A-HLF ACTS THROUGH LMO2 AND BCL-2 TO IMMORTALIZE LYMPHOID PROGENITORS

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The t (17;19) (q22;p13) translocation occurs as a non-random translocation in approximately 1% of childhood B-lineage acute lymphoblastic leukemias. This translocation results in the formation and expression of the chimeric transcription factor E2A-HLF. The concept of "oncogene addiction"has been proposed to explain the observation that some cancers are dependent on a small number of genes, or even a single gene,

for both maintenance of the malignant phenotype and cell survival. For instance, switching off expression of the BCR-ABL oncogene in mice reverses the leukemic phenotype. Therefore, inhibiting the activity of one or two key target genes required for the leukemic phenotype could inhibit cancer cell growth which could translate to improved outcome. These targets can either be activated oncogenes or their downstream targets. The "oncogene addiction"model also implies that overexpression of a few key downstream targets, of an initiating oncogenic lesion, could recapitulate the cancer phenotype. Therefore, the identification of genes that have altered expression in leukemogenesis by E2A-HLF and which are directly involved in tumor initiation and maintenance will be instrumental for identifying crucial therapeutic targets. We identified several novel targets of E2A-HLF, in particular Lmo2 and Bcl-2, the co-expression of which was sufficient to immortalize HPCs with a pre-B cell phenotype similar from that caused by E2A-HLF expression alone. Retroviral-mediated shRNA knockdown of Lmo2 expression or inhibition of BCL-2 family function both have severe consequences for the survival of E2A-HLF immortalized cells. Overall these data suggest that targeting of specific downstream targets of E2A-HLF is feasible and may provide a novel approach to treating patients with t (17;19) leukemia, which has hitherto proved fatal.

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POINT MUTATIONS IN NOTCH1 AND FBXW7 AND INTERNAL TANDEM DUPLICATION (ITD) IN FLT3 GENE IN POLISH PATIENTS WITH T CELL ACUTE LYMPHOBLASTIC LEUKEMIA (T-ALL)

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Background. T cell acute lymphoblastic leukemia accounts for about 15% of pediatric acute lymphoblastic leukemia cases. In Poland, about 40 children are diagnosed with T-ALL every year, and among those children as many as 30% relapse. This, relatively high failure rate is partly caused by the molecular and genetic heterogeneity of this disease. Many molecular genetic factors are suspected to contribute to clinical aggressiveness and response to treatment and among them mutations in genes controlling T cell maturation and proliferation seem to be the most promising targets for risk group classification and application of targeted therapies. *Aim.* The study was focused on the analysis of mutations in NOTCH1, FBXW7 and FLT3 genes in a group of Polish T-ALL patients. *Patients and Methods.* The study group consisted of 60 consecutive children with T-ALL treated at the centers of Polish Pediatric Leukemia and Lymphoma Study Group (PPLLSG). The mutation analysis was focused on exon 9 and 10 of FBXW7, exon 26, 27, 28 and 34 of NOTCH1 and was performed by specific amplification of regions of interest by PCR and followed by direct sequencing. The internal tandem duplication (ITD) of FLT3 was screened by PCR amplification of the regions of interest and fragment size assessment by agarose gel electrophoresis. *Results.* In total, we have detected 36 mutations in the whole group of T-ALL patients. Among those 36 events, 34 led to change of amino acid sequence (2 were synonymous). The mutations of FBXW7 were detected in 8% of patients, mutations of NOTCH1 were detected in 41.6% of patients, FLT3-ITD in 1.6% of patients. *Conclusion.* The frequency of detected mutations is slightly lower (12% for FBXW7 and 56% for NOTCH1) than reported by other authors from different countries. FLT3-ITD is rare phenomenon in pediatric T-ALL. We aim to continue our study in a larger group of patients to verify these observations and to evaluate clinical and prognostic significance of these molecular data after longer follow-up.

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VASCULAR ENDOTHELIAL GROWTH FACTOR-C AND ITS RECEPTOR TYPE-3 WERE EXPRESSED IN TWO CASES OF ACUTE LYMPHOCYTIC LEUKEMIA WITH T(1;19)

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Background and Aims. Vascular endothelial growth factor (VEGF)-C and VEGF receptor type-3 (VEGFR-3) play an important role in creation of systematic lymphatic vessels. VEGF-C and VEGFR-3 also play an important role in progression of malignant solid tumors; however, on our knowledge, there have been no reports on the relationship of acute lymphocytic leukemia (ALL) and VEGF-C system. We analyzed *de novo* ALL blasts to clarify whether VEGF-C and VEGFR-3 influenced ALL-pathogenesis. *Materials and Methods.* Bone marrow cells were obtained from informed ALL patients, whose mononuclear cells were prepared with density-gradient sedimentation method. Cells were cultured short term for the elimination of an adherent cell-fraction. RNA was extracted from the prepared non-adherent mononuclear cells, and the expression of VEGF-C and VEGFR-3 was analyzed with reverse transcription-polymerase chain reaction (RT-PCR). On the protein level, cells were analyzed with fluorescent activated cell sorter, and VEGF-C levels in patients' sera and in the conditioned media from the cultured ALL blasts were assayed with ELISA kit (R & D Systems, USA). When ALL blasts expressed VEGFR-3 and VEGF-C, a growth-inhibition effect by the administration of anti- VEGF-C-neutralizing antibody (Sigma, USA) was assayed with Cell-Counting Kit (Dojindo, Japan). *Results.* In 21 cases examined, VEGF-C production was observed in 4 cases. Of these four, VEGFR-3 was expressed in 2 cases, whose karyotypic analysis of the bone marrow cells showed t(1;19), and demonstrated E2A-Pbx1 fusion molecule with cDNA sequence (Applied Biosystems, USA). When anti-VEGF-C-neutralizing antibody was added to the blast cell-cultures, the cell-growth was significantly inhibited. *Conclusion.* These observations indicate that VEGF-C autocrine system works on proliferation in ALL with t(1;19).

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IN VITRO PROLIFERATION DYNAMICS OF GLUCOCORTICOID-TREATED CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) CELLS

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Background. Glucocorticoids are the most potent anti-inflammatory agents, widely used in lymphoid malignancies. Resistance or sensitivity to glucocorticoids is considered to be of major importance for the prognosis of leukemia, especially in lymphoid leukemia. While little is known on resistance to glucocorticoid-induced apoptosis, the proliferation dynamics governing leukemic cell systems under glucocorticoid influence could possibly elucidate the mechanisms leading to resistance. *in vitro* models present a powerful tool for the study of drug influence and have been utilized extensively in the study of glucocorticoids. *Aims.* Our previous work[1] elucidated that glucocorticoids can evoke mitogenic along with cytotoxic effects in leukemic blast cells. Cell viability, apoptosis, necrosis as well as DNA content have been studied. Attention has been drawn to the proliferation dynamics describing those cell populations. *Materials and Methods.* The childhood T-cell ALL CCRF-CEM cell line was utilized for the *in vitro* system. Cell count was measured with a NIHON KOHDEN hematocytometer. Cytotoxicity and DNA content were studied with flow cytometry on the FC500 Flow Cytometer (Beckman Coulter Inc.). Curve approximation was performed either linearly or quadratically ( ). Statistical analysis was performed using Microsoft Excel and Matlab® (Mathworks Inc.). Early apoptosis was calculated by using gating with Boolean functions. *Results.* Lower prednisolone concentrations increased cell growth speed, (Figure 1A), where N is the cell population measured at time t. Cells treated with lower prednisolone concentrations, started at a higher initial cell population (average: 1.8×10^3/uL). To test the possibility that growth speed was higher due to the initial cell population we per-

formed an independent experiment with no glucocorticoid addition, starting from ~2.8_103 cells/ul (Figure 1A). It appeared that increased proliferation was due to prednisolone treatment. Viable cells followed an expected pattern of proliferation with a<0 for all concentrations, where a is the quadratic coefficient (Figure 1B). Total cell death manifested a positive correlation as a function of time and concentration (Figure 1C, a>0) (Pearson's correlation 0.97). Different patterns of proliferation were revealed, when total cell death was discerned into its subcategories. Necrotic cells appeared to be significantly higher at low prednisolone concentrations as compared to untreated cells (Figure 1D, a>0). For 10nM and 1uM treatments (a<0) opposing proliferation patterns were observed as compared to higher doses and untreated cells (a>0) (Figure 1E). The same applied to early apoptotic cells (Figure 1F). *Conclusions.* Prednisolone manifested differential proliferation effects with mitogenicity at low concentrations and cytotoxicity at higher concentrations. This, could be partly explained by the differential proliferation of cell sub-populations i.e. the progression of viable, apoptotic, necrotic and early apoptotic cells. Proliferation dynamics could provide a useful tool for designing more efficient therapeutic protocols and our results could play a major role in determining prednisolone dosage in childhood ALL patients, as the response to glucocorticoids during the first week of treatment is considered to be crucial in the BFM-ALL protocol.

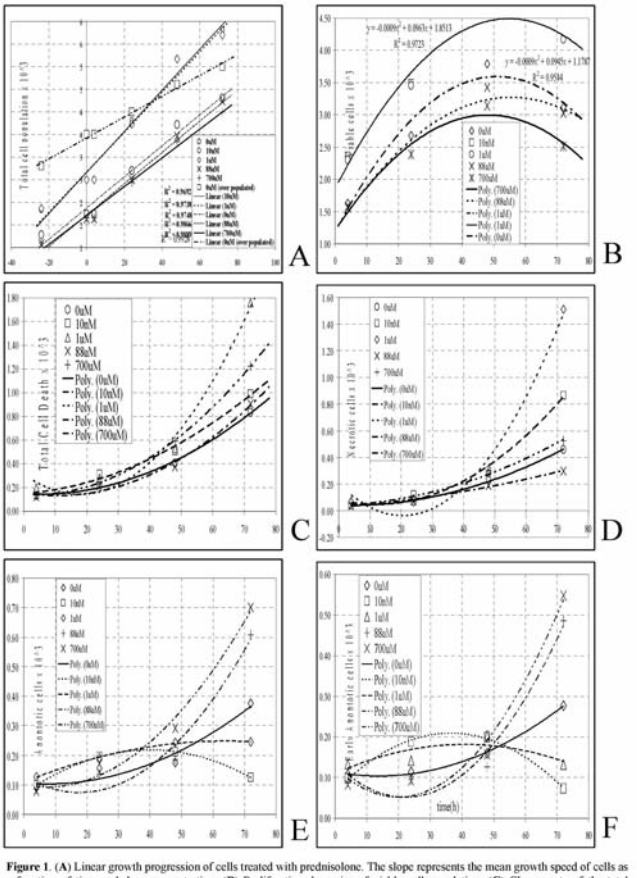

Figure 1. (**A**) Linear growth progression of cells treated with prednisolone. The slope represents the mean growth speed of cells as a function of time and drug concentration. (**B**) Proliferation dynamics of viable cell population. (**C**) Change rate of the total cytotoxic effect. Growth curve has been approached quadratically with $R^2>0.9$ for all concentrations. (**D**) Progression of necrotic cells during prednisolone treatment. (**E**) Population of cells undergoing apoptosis and similarly early apoptosis (**F**).

Reference

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PREVALENCE OF THIOPURINE METHYLTRANSFERASE GENE POLYMORPHISM IN EGYPTIAN CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. There is wide inter-individual variability in the pharmacokinetics, pharmacodynamics and tolerance of anticancer drugs. Polymorphisms that reduce the activity of thiopurine S-methyltransferase (TPMT) cause adverse reactions to conventional doses of thiopurines including 6-mercaptopurine (6MP), routinely used as anti-leukemia treatment. There are more than 20 variant alleles of TPMT that cause decreased enzymatic activity. *Aim.* To determine the frequency distribution of the most common variant alleles of Thiopurine methyltransferase (TPMT) gene polymorphism in a cohort of Egyptian children with acute lymphoblastic leukemia (ALL) and its correlation with response to chemotherapy and toxicity of 6MP. *Methods.*The study included 70 ALL patients diagnosed and treated in the Haematology /Oncology Clinic, Children's Hospital, Ain Shams University, Egypt during the period from January 1st 2004 till December 31st 2009, and followed up for a mean of 2.9±1.2 years. They ages ranged between 2-15 years (mean 6.16±4.3 years), with a M:F ratio of 2:1.6.The ALL treatment protocol was modified CCG 1991 (standard risk ALL), and modified CCG 1961 (high risk ALL). Patient's files were revised for patient's data at diagnosis including age, gender, ethnicity, initial total leucocytic count, immunophenotyping , hepatitis B or C infections; data about infectious complications including hospital admission, hepatic toxicity , neutropenia were recorded. Detection of TPMT gene mutation polymorphism was done using PCR technique followed by RFLP analysis. Three common alleles were examined:G238C, G460A and A719G. Seventy healthy age and sex matched children served as controls.*Results.* Neither the studied leukemic patients nor the controls had the TPMT variant alleles in either homozygous or heterozygous form. None of our patients had experienced relapse. 26.9% had T lineage immunophenotype and 73.1% had a pre-B phenotype. 50% of patients experienced at least one attack of febrile neutropenia which required hospital admissions. Infection necessitating hospitalization during the course of chemotherapy occurred in 62%(once), 6.3 %(twice) and 3.1% (more than 3 times). Liver enzymes were mildly elevated in 40.6% of patients and were elevated more than 200 IU/L in 13.3% and all of them had evidence of viral hepatitis. As a result of neutropenia and serious infections, patients needed 6-MP dose modification once in 53.1% , twice in 15.6%, 3 times or more in 6.3% , while 18.8% had no dose modification. Dose interruption occured in 62.5% of patients: 28.1% had 2 weeks interruption and 6.3% had more than 2 weeks interrupted therapy.*Conclusion.* TPMT variant alleles type G238C, G460A and A719G were not present in the studied patients, so genetic polymorphism in TPMT is not one of the determinant causes of 6-MP toxicity. The important causes of dose modification and interruption were infections and febrile neutropenia (environmental factors). So causes for chemotherapy interruption or modification other than TPMT gene polymorphism should be considered in Egyptian ALL patients.

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THE CASE REPORT: MINIMAL RESIDUAL DISEASE MONITORING USING PATIENT-ASSOCIATED ANTIBODY PANEL IN A PATIENT WITH MLL+ ACUTE B-LYMPHOBLASTIC LEUKEMIA

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Background. MRD monitoring by flow cytometry aims to identify cells with aberrant or rare phenotype. One of the approaches is to use patient-associated panels for MRD monitoring, which include (i) phenotypic markers of transformed cells identified at the date of a primary diagnostics and (ii) markers, often occurring in a particular variant of ALL. Immunological MRD monitoring is an individualized laboratory diagnostics, since individual patients can have a complete preservation of an aberrant phenotype, as well as the changes in the expression of several markers. *Aims.* We present a case of MRD monitoring using the patient-associated antibody panel in a patient with MLL+ acute B-lymphoblastic leukemia. *Materials and Methods.* The patient (female, 3 months old), came to the First Children State Hospital to the hemato-

logic department in July 2008. Analysis of the bone marrow samples by five-color flow cytometry was performed in Pavlov State Medical University. There were 6 points for MRD detection during monitoring of the disease. *Results.* According to the results of immunophenotyping by flow cytometry at the day of primary diagnostics, the bulk of bone marrow cells was represented by a homogeneous population of $CD19^+CD34^+CD22^+CD38^+CD33^+CD16^+CD13^+$ B cells. Molecular biological studies identified MLL-AF4. The aberrant expression of antigens CD33, CD13 and CD16 was assumed as a basis for the patient-associated algorithm of MRD monitoring. Because of the five color limitation, the antibody panel was formed in such a way, that the expression of myeloid markers could be evaluated on the $CD19^+CD34^+CD22^+CD38^+$ B-cells. At the day +15 from the beginning of the therapy transformed cells were revealed in amount 1,68%. Expression of CD33 remained stable, expression of CD16 decreased and expression of CD13 increased. At the day +36 expression was the same with 0,13% of the transformed B cells. At the day +43 expression of CD117 was newly revealed on the transformed B cells (0,2%) with decreased expression of CD33, CD13 and CD16. At the day +83 MRD was 0,11% confirmed by detection MLL-AF4. Expression of CD33 and CD117 remained stable and expression of CD13 and CD16 decreased again. MRD was more than 0,1% at the most of points, that means a high risk group. At the last point before the bone marrow transplantation (day +139) the tumor B-cells were revealed as 0,03%. Expression of CD33 and CD117 remained stable and expression of CD13 increased. *Summary*. During the MRD monitoring in the patient with MLL^+ acute B-lymphoblastic leukemia the aberrant expression of CD33 was stable. Other markers such as CD13, CD16, CD117 were not stable on the transformed cells. CD117 was newly revealed during the MRD monitoring. Expression of CD16 decreased with the beginning of the therapy and then disappeared at all. CD13 was firstly revealed only on a small part of transformed B cells. When the clone reduced, the expression of CD13 increased. Thus B cells with coexpression of CD13 could be more resistant for the chemical therapy. Results of flow cytometry using patient-associated antibody panel agreed accurate with the results of molecular biological studies and clinical data.

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ADULT T-CELL LEUKEMIA/LYMPHOMA ASSOCIATED WITH HUMAN T LYMPHOTROPIC VIRUS TYPE 1, REVEALED BY A SEVER HYPEREOSINOPHILIC SYNDROME: A CASE-REPORT

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Background. Hypereosinophilia may be secondary to a malignant lymphoid hemopathy. The multiple manifestations can be sudden, dramatic and potentially fatal. *Aims.* We present here a case of secondary eosinophilia with cardiac, neurologic and pulmonary complications, revealing an Adult T-cell Leukemia/lymphoma. *Methods*. A case-report *Results*. Ms S., 55 years old, living in Reunion Island (Indian Ocean), had a major hypereosinophilia (2 giga/liter) for 18 months, with only a diffuse skin rash. Parasitic, allergic and toxic etiologies are initially excluded. The patient is hospitalized in the intensive care unit with a multivisceral failure secondary to eosinophilic infiltrates in the myocardium, the lungs and the central nervous system. On the hemogram, we found a major hyperleukocytosis with a count of 74 giga/liter, 53 giga/liter of eosinophils and 9 giga/liter of lymphocytes. The diagnosis of Adult T-cell Leukemia/lymphoma was made by the presence of clonal lymphoid T cells (positive T-Cell Receptor Gene rearrangement) with an aberrant phenotype, the presence of Sezary-like cells CD25 positive and the positive serology of Human T Lymphotropic Virus type 1. The FIP1L1/PDGFRA and BCR-ABL mutations are negatives; the medullar karyotype is normal; an eosinophilic infiltrate was found in both bone marrow biopsy and aspiration, and a dermal superficial lymphoid T infiltrate in the skin biopsy. All the symptoms of hypereosinophilia disappeared after few days of corticoid pulse therapy associated to Hydroxyurea, with normalisation of the hemogram. So, the specific treatment of Adult T-cell Leukemia/lymphoma by the association of Interferon alpha and Zidovudine was introduced. The mother of the patient, 96 years old, has been found to be positive for the Human T Lymphotropic Virus type 1 serology without any symptom. The mode of transmission of the virus was probably the maternal breast-feeding, with the first clinical symptoms appearing for the period known from 10 to 50 years later. Thus, the philogenetic study of this virus is ongoing. *Conclusion*. T malignant hemopathy and viral infections are known to be responsible of hypereosinophilia, mostly moderated and asymptomatic, governed by several cytokines such as IL-3 and IL-5, stimulating the production of eosinophil cells. So, due to a meticulous etiological investigation and to advances in the diagnostic techniques, secondary causes of eosinophilia can be identified in a proportion of cases which would have otherwise been classified as idiopathic hypereosinophilic syndromes and then can be specifically treated. The originality of this observation is the extremely severe impact of the hypereosinophilia, including in particular cardiac failure, more classically observed in the myeloproliferative forms of hypereosinophilic syndromes.

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THE AMMONIA CONCENTRATION EVALUATION AS THE ADDITIONAL METHOD FOR THE ASPARAGINASE ACTIVITY MEASUREMENT

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In the last years some authors have suggested, that the evaluation of ammonia concentration in serum could be an effective and simply way to the estimation of L-asparaginase activity during the treatment. The aim of the study was to evaluate the possible usability of the estimation of serum ammonia concentration as the method of the evaluation of L-asparaginase activity. *Material and Methods.* Fifteen patients with *de novo* ALL, treated with several doses of L-asparaginase (5000 IU/m^2, 10000 IU/m^2 and 25000 IU/m^2) were enrolled to the study. The blood samples were collected from each patient in two time points: before the administration of the drug and three days after the administration. The estimation of ammonia concentration was performed using the standard spectrophotometric method, L-asparaginase activity was established with ELISA method. *Results.* Ammonia concentration was strongly associated with the dose of L-asparaginase, the highest values were observed after the dose of 25000 IU/m^2. There was the significant association between the concentration of serum ammonia, estimated three days after drug administration and the serum L-asparaginase activity (R=0.495, P=0.019 The AUC value ammonted 0.794 with 95% confidence range of 0.57-0.934. The threshold ammonia value corresponding with L-asparaginase activity of 100 U/L, estimated using the ROC curve, amounted 66 µg/mL. The sensitivity of the method was assessed as 52.94%, the specificity as 100% , P=0.004. *Conclusion.* The method based on the measurement of ammonia concentration might be useful in case in which the measurement of L-asparaginase activity is not accessible.

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MINIMAL RESIDUAL DISEASE DETECTION IN CHILDHOOD B-LINEAGE ACUTE LYMPHOBLASTIC LEUKEMIA BY MULTICOLOR FLOW CYTOMETRY USING THE ST JUDE APPROACH

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Background. Minimal residual disease (MRD) is a powerful and independent prognostic factor in childhood acute lymphoblastic leukemia (ALL). This submicroscopic detection of residual blasts during therapy is typically performed by flow cytometry or polymerase chain reaction (PCR) amplification of antigen-receptor gene rearrangement. Currently, our center utilises PCR methodology as part of the routine assessment of therapeutic response. This study aims to evaluate the applicability of the St Jude flow cytometry approach to detect MRD in our patient cohort recruited in the Malaysia-Singapore ALL Study. *Methods.* Ficoll-purified mononuclear cells were obtained from 112 follow-up bone marrow/peripheral blood samples collected from 29 patients diagnosed with B lineage ALL (B-ALL). Institutional ethical committee approval and informed consent from parents were obtained. Leukemia associated antigens (LAA) were identified from diagnostic or relapse samples with 4 colour flow cytometry using fluorochrome-conjugated monoclonal antibodies to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), and allophycocyanin (APC). Discrimination of leukemic blasts was achieved by using one or more of the selected LAA stained with a backbone panel of antibodies consisting of CD10, CD19 and CD34. Those combinations that would allow identification of one leukemic cell in 104 or more normal nucleated bone marrow cells were selected and applied to the MRD samples.

Analysis was performed by comparing the staining pattern of each combination to that of 'normal' bone marrows. *Results.* Good concordance was obtained in 96% of the follow-up samples (P<0.001) when we compared dichotomous MRD status (positive/negative) by flow cytometry and PCR *Methods.* Overall, 58 samples were MRD negative (<0.01%) and 49 samples were MRD positive (≥0.01%) by both techniques. Of the samples that were MRD positive by both methods, 67.4% were within half log difference, 22.4% and 10.2% were within one log and 1-1.5 logs respectively. In the remaining 5 samples that were MRD<0.01% by flow cytometry but ≥0.01% by PCR, 5 were around the threshold level. *Conclusion.* A good correlation was obtained between MRD results by flow cytometric and molecular *Methods.* The use of both assays in tandem would enable us to monitor MRD in a larger proportion of patients with B-ALL as neither technique alone can be used to follow MRD in all patients. The methods in tandem will also reduce the chance of false negativity due to phenotypic shifts or clonal evolution.

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PEDIATRIC THERAPY IN ADULT ACUTE LYMPHOBLASTIC LEUKEMIA: EXPERIENCE OF A SINGLE CENTRE

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Background. Acute lymphoblastic leukemia (ALL) shows different outcome in children and adults, with event-free-survival (EFS) rates of 70-80% and 30-40% at 5 years, respectively. This reflects both a different disease biology and different therapeutic approaches. Recently, results apparently improved in young adults/adolescents aged 15-21 years, with *de novo* ALL, when treated with pediatric intensive regimens rather than with typical adult regimens. Similarly, clinical studies are ongoing in older patients, toxicity related-therapy seeming the limiting issue. *Aims.* We report a single centre experience on adult ALL patients treated with an intensive pediatric-inspired schedule, aiming to assess its tolerability and efficacy. *Methods.* From November 2007 to June 2009 eleven ALL patients (M/F=9/2) were treated at our Center according to modified AIEOP-LAL2000 regimen. Treatment consisted of 7 days steroid pre-treatment, and four drugs 78-days induction (phase IA and phase IIB) after which high risk (HR) patients were treated with three polychemotherapy blocks, while intermediate (IR) and standard risk (SR) patients went on 8-weeks consolidation and subsequent delayed intensification. Allo-SCT was planned for all patients with HLA-matched donor; 2-years maintenance therapy was given to the others. Median age was 31 years (range, 17-47). According to cytogenetic, response to steroid and minimal residual disease patients were classified into HR (n=5), IR (n=4) and SR (n=2). *Results.* 9/11 patients completed the induction phase IA, two being out for toxicity (grade IV infection and intestinal occlusion). Seven (64%) obtained a complete remission (CR); two were refractory. However, one of them subsequently achieved CR after polychemotherapy blocks, for an overall response rate of 73% (8/11). Seven patients then completed the 28-days induction IB. Median induction duration was 92 days (range 82-136). Delays were mostly due to extra-hematological toxicity, the commonest being gastrointestinal (n=12), infective (n=7) and thrombotic (n=3). Delays were accumulated in both induction phases without significant difference between phase IA (median 18.5 days, range 4-37) and phase IB (median 17 days, range 9-66), despite an absolute number of moderate-severe AE superior in phase-IA *vs.* phase-IB (12 *vs.* 5). After induction, 3/8 patients received consolidation therapy; 2/3 then received allo-SCT. The median duration of consolidation was 51 days (range 22-94). Conversely, 5/8 patients received polychemotherapy-blocks: 1/5 dropped out after one block due to reversible grade III renal failure. All the other four received allo-SCT. The median CR duration was 17 months (range 7+-35+); two patients relapsed, both after allo-SCT. With a median follow-up of 23 months (range 8-36) 8/11 (73%) patients are alive, 7 in CR (4 undergone allo-SCT). Three patients dead, one in CR for infection after allo-SCT, 2 for relapsed/refractory disease. *Conclusions.* Though in a small series, pediatric-like intensive chemotherapy seemed to be feasible in adult ALL. Extra-hematological toxicity, however, caused significant treatment delays during induction. Finally, the overall outcome appeared promising, though longer follow-up and larger populations are needed to draw definitive conclusions.

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MRD-BASED TREATMENT STRATEGY FOR CHILDHOOD ALL (PROSPECTIVE STUDY OF 62 PATIENTS: TUNISIAN EXPERIENCE)

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Background. In a previous retrospective analysis of 58 children with ALL treated according to the EORTC 58951 protocol between 2001 and 2003, patients were stratified by , WBC at diagnosis, immunophenotype, karyotype and steroids response in peripheral smears after 7 days of corticosteroid and one IT dose of MTX. 3-year EFS and OS were unsatisfactory: 46% and 55.4%. In order to improve our *Results.* blast cell count in the BM on D7 and D19, and determination of MRD measured by flow cytometry (CMF) in the BM at D35 were included in our stratification strategy. Patients whose blast cells were ≥25% in BM (M3 marrow) at D7 and or at D19, or whose MRD was positive at the end of induction, had their treatment one step upgraded. The aim was to determine the prognostic impact of morphological assessment of BM blasts on D7 and D19 during remission induction and the prognostic impact of the presence of MRD after remission induction. *Methods.* from January 2006 to December 2007; 62 children (1.5 years - 21 years) with newly diagnosed ALL were treated according the protocol of EORTC 58951 modified. We performed a prospective study of an early treatment response as assessed by morphological examination of BM blasts on D7,D19, and MRD determination by CMF, based strategy. All patients received phase IA with HD-MTX (D8) and cyclophosphamide (D9). 19 patients were treated according to the EORTC 58951 AR2 arm and 35 enrolled to the VHR protocol. *Results.* 62 pediatric patients (37 males and 25 females) were enrolled, 37,1% aged ≥10 years at diagnosis, 32% with a WBC ≥50 Giga/L. Immunophenotyping was done for all patients: 40 (64,5%) B-ALL, 17 (27, 4%) T-ALL, 1 undifferenciated and no contributive in 4 cases. 3/40 (7,5%) B-ALL were steroid poor responders *vs.* 12/17 (70,5%) with T-ALL; P<0.001. 42 (67.7%) patients had M3 marrow (>25%) responses on D7. On D19, 4 patients had M3 marrow. CR was 84% (52/62), induction failure and death rates were 3% (2/62) and 13% (8/62) respectively. MRD results available in 45 cases, sensitivity of 0,01%, between 0,01% and 0,1% , between 0,1% and 1% and ≥1% was achieved respectively in 33%, 27%, 20% and 20%. 3-year EFS and OS were 62.6±6.7% and 73.4%±6%. MRD < 10^{-4} at the end of induction had significantly better outcome than those with a level ≥10^{-4} (3-year EFS: 81.5%±11% if MRD <10^{-4} *vs.* 68,2%±15% if 10^{-4}<MRD≤10-3 *vs.* 77.8±13% if 10^{-3}<MRD≤10^{-2} *vs.* 37%±18% if MRD>10^{-2} , P:0.004). *Conclusion.* the treatment results of this cohort were significantly better than those of previous cohorts in terms of 3-year EFS (62.6% *vs.* 46%) and 3-year OS (73.4% *vs.* 55.4%). Early treatment as assessed by morphological examination or MRD based treatment strategy seems to be highly successful and may improve the outcomes of children with ALL in terms of relapse. However the induction death rates increased.

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OXIDATIVE STRESS, ANTIOXIDANT STATUS AND THEIR RELATION TO APOPTOSIS IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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Introduction. Oxidative stress may play a role in the development of carcinogenesis. It is well established that some chemotherapeutic agents and radiation therapy generate reactive oxygen species (ROS) in patients during cancer therapy. Acquisition of mechanisms to evade apoptosis is a hallmark of cancer. Defective apoptotic mechanisms allow genetically unstable cancer cells to avoid elimination and confer resistance to chemotherapy. Apoptosis and cancer are opposed phenomena, but ROS may play a key role in both. *Aim of the work*. Evaluate oxidative stress (malondialdehyde (MDA) and total antioxidant capacity (TAC)) and apoptosis level in childhood ALL at diagnosis and their impact on outcome at the end of induction of remission phase. *Patients and Methods.* This study included 50 newly diagnosed children

with ALL. They were 29 males and 21 females with age mean±SD (6.84±3.73 year). Evaluation of oxidative stresses (MDA and TAC) at diagnosis and after the end of induction phase. Apoptosis was evaluated by Fluorometric TUNEL System for patients at diagnosis and after one week of treatment. *Results.* There was a significant increase of oxidative stress in children with ALL at diagnosis compared to controls (MDA: 16.61±6.55 nmol/mL *vs.* 6.42±1.52 nmol/mL, and TAC: 0.88±0.33 mM/L *vs.* 1.57±0.36 mM/L) (P<0.001). Also there was further significant increase in oxidative stress in the same patients after 5 weeks treatment with chemotherapy compared to the level at diagnosis (P<0.001). Apoptosis index was found to be significantly higher after one week treatment with chemotherapy when compared to its level at diagnosis (26.58±14.06 % *vs.* 88.45±12.11 %). Oxidative stress was found higher at diagnosis in patient who died before completion of induction. A lower apoptotic index at diagnosis was found among patients who were resistant to treatment in comparison to responsive patients. There was a significant negative correlation between TAC and MDA at diagnosis (r=-0.461, P=0.001) and after treatment (r=-0.518, P=0.001). Also, there was a positive correlation between level of oxidative stress at end of treatment and apoptosis index after 1 week. *Conclusion.* There is increased oxidative stress in children with ALL, increased ROS or decreased antioxidant capacity, which may participate in leukemia pathogenesis. Chemotherapeutic agents may further increase oxidative stress and apoptosis in ALL. Apoptotic index may serve as a predictor for response to chemotherapy. Level of apoptosis after therapy may be correlated with level of oxidative stress.

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CHROMOSOMAL ABNORMALITIES IN ACUTE LYMPHOBLASTIC LEUKEMIA (ALL): KARYOTYPE AND FISH STRATIFIED WORK UP

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ALL constitutes a heterogeneous group of diseases resultant from the clonal expansion of lymphoid progenitors. The diagnosis is established by morphology, flow cytometric immunophenotyping and identification of chromosomal and molecular abnormalities. Different ALL subtypes have distinct response to therapy as well as diverse prognosis. Adults and children with the same genetically defined subtype can have markedly different responses to therapy and prognosis. Thus, patient age and cytogenetics are extremely important variables to stratify risk and to select therapy. The present study was conducted to evaluate the rate of chromosomal abnormalities in ALL patients diagnosed in Brazil, and to compare the rate of cytogenetically defined subtypes of ALL between adults and children in a central laboratory in São Paulo, which receives samples from the whole country. From January 1997 until December 2005 we identified 173 consecutive cases with available results from morphologic, immunophenotyping and cytogenetic tests. There was a 1.5:1 male-to-female rate; age varied from less than 1yr-old till 87yr, with the following distribution: 4% with <1 year-old; 56% from 1 to 10yr; 22% from 11 to 25yr; and 18% from 26 to 87yr. Thus, 60% of the patients were children (<10 yr), with median age of 8 years, and 40% were teenagers or adults, with median age of 47 years. Chromosomal abnormalities were detected in 80% of the cases, as follows: pseudodiploid: 15%; 47-50 chromosomes: 9%; 51-60 chromosomes: 23%; >60 chromosomes: 3%; hypodiploid: 4%; Philadelphia: 6%; t(4;11): 5%; del(11q23): 2%; t(1;19): 8%; t(8;14) or variants: 2%; Down syndrome-ALL: 2%; no *Results.* 3%. Comparing aberrations between children/teenagers (<20 years) and adult patients, the following statistically significant differences (P<0.05; student t-test) were observed: high hyperdiploid: 31% *vs.* 9%; Philadelphia: <2% *vs.* 27%, and t(1;19), 8.8% *vs.* 0%, respectively. Karyotype is considered an independent variable for ALL prognosis and allows the stratification of patients for different therapeutic options. The rate of success observed in karyotype results (80%) was satisfactory, with less than 3% of failures. Pullarkat *et al.*, (2008) have found 70% of cytogenetic evaluable results among 200 ALL cases from 15 to 60 year-old patients in the SWOG study. The Philadelphia chromosome was more frequently detected among adult patients in the present study, as previously shown by others (Pullarkat *et al.*, 2008), while the t(1;19) was not detected in adults. High hyperdiploid karyotypes (51-60 chromosomes) was frequent among children, as expected. The percentage of normal cases was 21.6% and 20% for children and teens/adults, respectively, similar to others (Pullarkat *et al.*, 2008). As fluorescent *in situ* hybridization (FISH) is still an expensive method for routine use in developing countries, the strategy designed was to add FISH for the investigation of chromosomal abnormalities only in patients with normal karyotype. A subsequent analysis with FISH using probes for ALL (Chromoprobe Multiprobe ALL-System,Cytocell, England) in 19 ALL cases with normal karyotype identified abnormalities in 9 of them, involving the following genes: CHIC2, P16, IgH, E2A and AML1. Therefore, this stratified work up is a cost-effective approach to detect clinically relevant genetic abnormalities in ALL.

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TREATMENT OF PHILADELPHIA-POSITIVE ADULT ACUTE LYMPHOBLASTIC LEUKEMIA IN REAL LIFE: EXPERIENCE OF ONE CENTRE

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Background. Philadelphia chromosome positive acute lymphoblastic leukemia (Ph+ ALL) in adult patients is associated with a particularly unfavorable prognosis, although some progress has been made recently with the use of tyrosine kinase inhibitors (TKIs). *Aims.* Assessment of outcomes of Ph+ ALL patients treated with or without a TKI in one hematological center. *Methods.* Twenty-four consecutive patients with newly diagnosed Ph+ ALL were included in the analysis, 14 (58%) patients were treated with chemotherapy and a TKI (imatinib in all cases), 10 (42%) with chemotherapy alone, mostly in the pre-TKI era. Eleven (46%) patients underwent stem cell transplantation (SCT); 8 matched unrelated donor SCT, one HLA identical sibling donor SCT and 2 autologous SCT. Data were evaluated for complete hematologic remission (CR), complete molecular remission (CMR) and relapse rates and for risk factors affecting overall survival (OS). We were mostly interested in the effect of adding a TKI to chemotherapy. *Results.* Out of the total number of 24 patients, 19 (79%) achieved a CR (median time to CR 37 days, range 6-68 days), 12 (75%) of 16 evaluable patients achieved a CMR (median time to CMR 67 days, range 32-358 days). Nine (47%) patients experienced a relapse (median time to relapse 7.6 months, range 1.1-37.5 months) with a very short post-relapse survival regardless of the type of salvage therapy. Molecular relapse was in all but one cases followed by a hematological relapse (median time to molecular relapse 2.9 months, range 1.4-13.1). At the end of the follow-up period with a median of 8.7 months, 8 (33%) patients were still alive in CR (only one treated in the pre-TKI era) and 16 (67%) died, mainly of infection (9 patients, 56%) and disease progression (5 patients, 31%). Median OS was 9.2 months; the highest survival benefit was seen in patients achieving a CR (P<0.001) or a CMR (P=0.026) and treated with allogeneic SCT (P=0.003). The addition of a TKI to chemotherapy was associated with a higher CR (P=0.075) and CMR rate (P=0.05), but neither with a lower relapse rate (P=0.26) nor a prolonged OS (P=0.13). *Summary/Conclusions.* According to our analysis, in patients with Ph+ ALL the best outcome is achieved with the combination of chemotherapy, TKI and allogeneic stem cell transplantation.

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SECOND GENERATION TYROSINE KINASE INHIBITORS CAN INDUCE COMPLETE MOLECULAR RESPONSE IN PH-POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA RELAPSING AFTER ALLOGENEIC STEM CELL TRANSPLANT

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Background. Allogeneic stem cell transplant (SCT) is thought to be the only curative therapy for Ph+ acute lymphoblastic leukemia (ALL). New therapeutic strategies, including combination of tyrosine-kinase inhibitors (TKI), chemotherapy and SCT may improve results in terms of complete remission and disease free survival. Dasatinib was highly active both in imatinib-resistant patients and as front-line treatment. Nilotinib has also shown activity in Ph+ALL in phase I studies. Few cases of successful dasatinib and nilotinib therapy in Ph+ALL relapsing after BMT have been reported, either in adults or pediatric patients *Aims.* We report two cases of Ph+ALL relapsing after allo-SCT who obtained a complete response with II generation TKI. *Results. Case 1.* A 21-year-old man was diagnosed of Ph+ALL in March 2006. He was administered chemotherapeutical treatment with Hyper-CVAD plus imatinib, obtaining a complete haematological response (CHR) and complete cytogenetic response (CCyR) maintained over treatment while major molecular response (MMR) was never achieved. Eight months after diagnosis the patient was submitted to a SCT from match unrelated donor (MUD)

with myeloablative conditioning (TBI and cyclophosphamide). Three months after transplant the disease relapsed. Treatment with dasatinib was started at 70 mg bid. Blast clearance in PB was obtained in 5 days, and was accompanied by a tumor lysis syndrome. CHR was achieved after 1 month, and complete molecular response (CMR) was obtained after 4 months of therapy. Donor Lymphocyte Infusions (DLI) were administered every 6 weeks for a total of 5 infusions. No signs of GVHD were observed. Due to a grade 2 diarrhea, dasatinib dosage was reduced to 120 mg/day. The patient is in good clinical conditions and bone marrow is persistently PCR negative 34 months after relapse. *Case 2.* A 18-years-old boy was diagnosed as Ph+ALL in December 2008. He was treated with high dose chemotherapy and imatinib, obtaining a CHR with persistence of molecular disease. In May 2009 he underwent a allogeneic MUD transplant after TBI and cyclophosphamide. In spite of complete engraftment and BCR/ABL transcript reduction (not reaching MMR), he relapsed in October 2009. He started treatment with dasatinib 70 mg bid, resulting in blast clearance and recovery of 93% chimerism at day 15. A single dose of DLI was administered at day 60. After 3 months a full donor chimerism with <0.1% blasts was observed in the bone marrow. The patient however complained of diarrhea G3 and dasatinib was stopped at month 4, when nilotinib 400 mg bid was started. Diarrhea resolved and the patient is well and in CMR five months after relapse. *Conclusions.* Our patients showed a prompt response to dasatinib treatment. In one case, it was associated with DLI infusion, and CMR is stable 34 months after relapse, with full donor chimerism. In the second case, dasatinib therapy was accompanied by gastrointestinal toxicity which resulted in change to nilotinib, with persistent disease response. Second generation TKI can rescue Ph+ ALL patients after SCT failure. Role of DLI in this setting is unclear, but warrants further investigation.

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ACQUISITION OF THE NOVEL COMPOUND ABL KINASE DOMAIN MUTATION T315L IN A RELAPSED PHILADELPHIA-POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA PATIENT

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Background. The incorporation of the tyrosine kinase inhibitor imatinib mesylate for frontline treatment of Philadelphia-positive acute lymphoblastic leukemia (Ph^+ ALL) has significantly improved the antileukemic efficacy of induction therapy. Several ALL study groups have demonstrated complete remission rates above 90% in patients undergoing imatinib-containing therapeutic regimens and improved long-term outcome in these patients. Despite such improvement, however, relapse does occur largely due to acquisition of imatinib resistance. Although numerous point mutations in the ABL kinase domain that impair imatinib binding have been identified as a major mechanism of acquired resistance in CML patients, similar data on Ph^+ ALL patients are more limited. Here, we describe a case in which a novel ABL kinase domain mutation was detected at relapse in a patient with Ph^+ ALL. *Case report.* A 50-year old male was diagnosed with acute lymphoblastic leukemia at our institution in January 2009. His bone marrow karyotype was 46,XY,t(9;22)[15]/ 47, idem,+der(22)t(9;22)[4],46,XY[1]. Reverse transcriptase polymerase chain reaction (RT-PCR) demonstrated presence of the p190 BCR-ABL fusion transcript. He underwent chemotherapy with hyperCVAD-imatinib mesylate. Complete morphological and cytogenetic remissions were achieved as documented by bone marrow studies performed after 2 rounds of chemotherapy, following complete recovery of white cell and platelet counts. Molecular studies did not detect the p190 fusion transcript in 2 consecutive peripheral blood specimens collected in March and April 2009. However, following the sixth cycle of chemotherapy in September, he was noted to have 3% blasts in his peripheral blood and reappearance of p190 BCR-ABL fusion transcript. Bone marrow aspirate revealed relapsed ALL. Cytogenetic studies revealed recurrence of the Philadelphia clone. Mutation analysis to detect ABL kinase domain mutations was performed on both peripheral blood and bone marrow specimens collected on relapse. Both demonstrated the presence of a novel ABL kinase mutation, T315L, which has not been previously described in the literature. This mutation was absent in the bone marrow specimen collected at diagnosis. Mutational analysis of the ABL kinase domain was performed using direct sequencing approach. Amplified products were sequenced in both forward and reverse directions. Nucleotide changes were observed at positions 943A>C and 944C>T (Genbank NM_005157.3 CDS), resulting in a T315L mutation. Cloning experiments confirmed the presence of the T315L mutation in a single clone. Therapy with dasatinib was initiated. The patient failed to achieve remission after 2 months and the disease was refractory to chemotherapy. He subsequently died of fulminant sepsis. *Conclusions.* This is a unique case report detailing the finding of a novel compound mutation T315L detected at relapse in a p190 Ph^+ ALL patient which was associated with TKI resistance. Although the impact of a T315L mutation on the clinical efficacy of imatinib or dasatinib is not certain, it is likely that this mutant disrupts binding of TKIs in a way similar to the T315I mutant. Perhaps in-vitro studies to evaluate its grade of resistance towards all common TKIs could help to assess this further.

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ERWINIA ASPARAGINASE AS THE ALTERNATIVE USAGE FOR E. COLI ASPARAGINASE-ALLERGIC CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA IN KOREA

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Background. Escherichia coli (*E. coli*) asparaginase is an active drug of treatment for childhood acute lymphoblastic leukemia (ALL). However, the side effects of E. coli asparaginase develops very often. We inquired out the incidence of the side effects of E. coli asparaginase in Korea, and evaluated the safety of subsequent use of Erwinia asparaginase, an alternative preparation, in E. coli asparaginase-allergic patients. *Patients and Methods.* Between 2006 and 2009, medical records of 379 children with newly diagnosed ALL in 8 hospitals of South Korea were reviewed to inquire out the major side effects, which made the administration of L-asparaginase discontinued, including allergic reaction, pancreatitis, hemorrhagic event, and thrombotic event. *Results.* 1) Seventy-three patients(19.2%) developed the side effects of E. coli asparaginase. Sixty of 73 patients had allergic reaction. 37 patients had mild local reaction, but the 23 patients had systemic allergic reaction. Other E. coli asparaginase related side effects included pancreatitis(4 patients), coagulopathy (3 patients), and a hemorrhagic event(a patient). Side effects were more frequent in high risk group (55patients) than in standard risk group (19 patients). 2) Sixty-one patients switched to Erwinia. Among them, 4 patients had allergic reaction. 3 patients had local reaction, and a patient had systemic reaction. Only 4 patients(1%) from 379 patients had a allergic reaction to both type of E. coli and Erwinia asparaginase. *Conclusions.* Erwinia asparaginase was well tolerated and acceptable for E. coli asparaginase-allergic patients. E. coli asparaginase with subsequent Ewinia asparaginase in E. coli asparaginase allergic patients could be the way to overcome the sensitization by asparaginase antibody. Especially in high risk ALL, Erwinia asparaginase should be considered for E. coli asparaginase-allergic patients.

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FLT3 MUTATIONS IN MLL-REARRANGED INFANT ACUTE LYMPHOBLASTIC LEUKEMIA (ALL): EXPERIENCE OF A SINGLE PEDIATRIC INSTITUTION

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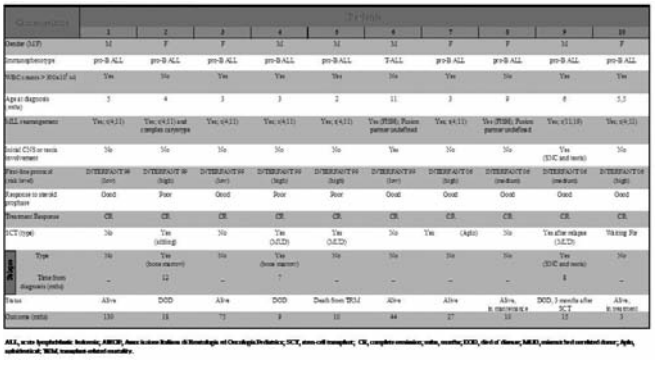
Background. Acute lymphoblastic leukemia (ALL) of infant (up to 1 years of age) is characterized by poorer prognosis compared with older children, high frequency of MLL-rearrangement and major resistance to chemotherapeutic agents. New therapeutic targets have to be identified for improving the outcome of MLL-rearranged ALL. Recent study have demonstrated mutations of FLT3 in approximately 15% of infants with MLL-rearranged ALL and various clinical trials with FLT3 inhibitors are under investigations. Besides highest levels of FLT3 mRNA expression have been reported in leukemic infant cells with MLL-rearrangement. Aim of the study was to assess the presence of FLT3/internal tandem duplications (ITDs) as well as D835 mutation of

FLT3 in infant ALL with MLL-rearranged treated in a single pediatric Institution. *Patients and Methods.* From January 2000 to January 2009, 10 infants (range, 2-11 months), with MLL-rearranged ALL, were enrolled in INTERFANT-99 and INTERFANT-06 protocols in our Institution. All patients, except one with a T-cell ALL, had a CD10- proB immunophenotype; 6 infants presented at diagnosis a WBC counts more than 300×10^3/mmc and two-thirds of patients were younger than 6 months. Table 1 resume the characteristics of patients. Primary bone marrow samples of patients were retrospectively studied for ITDs and D835 mutation and possible correlation between FLT3 mutations and prognosis was investigated. Genomic DNA of all patients was extracted from bone marrow aspirate using standard procedures. PCR fragments of FLT3 gene were amplified using the following oligonucleotide primers: ITD-Forward (ITD-F) (5'-TGTCGAGCAGTACTCTAAACA-3'), ITD-Reverse (ITD-R) (5'-ATCCTAGTACCTTCCCAAACTC-3'), D835-F (5'-AGAAGAGGAGGACTTGAATGTGCTTA-3'), D835-R (5'-TCCATATGACCAGACATCACTCTTAAT-3'). Internal tandem duplication as well as D835 mutation in the FLT3 gene were screened by DHPLC (denaturing high performance liquid chromatography; Wave System; MD Transgenomic Inc., Omaha, NE). Electropherograms from patients were compared with normal sequenced controls. The levels of FLT3 mRNA expression using quantitative real-time PCR were not performed. *Results.* None of the infant ALL harboured ITDs and D835 mutation of FLT3, so no correlations with prognosis could be found. Six infants are alive in CR (of whom 4 off-therapy and 2 in treatment) and 4 died (3 from disease and 1 from transplant toxicity). Five patients underwent an allogeneic transplant, of whom only one is alive in CR ; 3 infants relapsed after BMT and one died from TRM. Four patients were treated with chemotherapy alone and are alive in CR (1 is still in maintenance) and the last one is waiting for a mismatched unrelated donor (MUD) transplant. No patient was treated with FLT3 inhibitors. *Conclusions.* Our results are in accordance to previous studies indicating a lower incidence of FLT3 mutations in MLL-rearranged infant ALL. In our simple size sample, we analyzed only ITDs and D835 mutation of FLT3 in infant ALL. Probably other FLT3 mutations are involved in the ethiology of FLT3 expression. Since FLT3 inhibitors seem effective in MLL-rearranged infant ALL, further study are necessary to assess the potential impact of FLT3 in the pathogenesis of infant ALL.

Table 1. Clinical characteristics o infant ALL.



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TREATMENT OUTCOME OF ADOLESCENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA IN A TUNISIAN CENTER

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Introduction. Retrospective studies have shown that adolescents with acute lymphoblastic leukemia (ALL) treated with paediatric protocols have better outcomes than those treated with adult ones but with an increase in hematologic toxicity. *Purpose.* Comparison of the clinical characteristics, laboratory features, results of the treatment outcome and specially the toxicity of adolescent and younger children with newly diagnosed ALL treated at the department of hematology of Aziza Othmana Hospital. *Patients and Methods.* Between January 2005 and December 2006, 62 ALL up to 21 years of age were assessed and treated according to the EORTC 58951 paediatric protocol. The patients were subdivided in two groups according to their ages: -First group children who were <15 years old and the second group consisted of adolescents aged between 15 and 21 years old. *Results.* 11 patients were assessed in the second group (18%).There were no differences between the two groups with respect to gender, WBC count and CNS involvement at diagnosis. However the distribution of T ALL and the poor corticosteroid response were more frequent in the second group with respectively 46% *vs.* 23.5% (P<0.0001) and 54.5% *vs.* 19.6% (P=0.02). When adolescent and younger were compared to each other, there were no statistically significant differences in hematologic toxicity in induction, consolidation and reinforcement cycles, in CR (100%vs 89%), in relapse rates (27% *vs.* 18%), in OS (81% *vs.* 70%) and in EFS (61.4%vs 62.4%). *Discussion.* Data seem to indicate that adolescents with ALL have a similar prognostic to this of young children, despite predominance of T ALL and poor corticosteroid response, when treated with paediatric protocols without more toxicity. *Conclusion.* Adolescents and young adults with ALL might benefit from paediatric protocols

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AZACYTIDINE AS A NOVEL AGENT IN THE TREATMENT OF ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. 5-Azacytidine (AZA) is a hypomethylating agent with activity in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML), but not in acute lymphoblastic leukemia (ALL). The transformation of MDS into ALL, while far less common than transformation into AML, is a rare but known complication. *Aims.* We present the first case report of AZA with activity in ALL evolving from MDS. *Methods.* A 74-year-old woman initially presented in 2005 with moderate thrombocytopenia (83×10^9/L). An initial bone marrow aspirate (BM) was consistent with MDS (refractory cytopenia with multilineage dysplasia). She did not require chemotherapy or transfusions at that time. In January 2009, she developed worsening pancytopenia, and a BM showed an abnormal population of immature lymphocytes, with a pre-B cell phenotype (CD34, CD19, CD10, CD79a, and CD22 positive, and CD20 and surface immunoglobulin negative). This population represented 43% of all nucleated cells in the marrow by flow cytometry. She received therapy targeting the ALL, consisting of intravenous cyclophosphamide (750 mg/m^2), vincristine (1.4 mg/m^2), and oral prednisone. She tolerated therapy poorly, with a prolonged hospital admission for a febrile neutropenic episode. A second regimen consisting of 6-mercaptopurine (50 mg/m^2 given intravenously 14 days out of a 21 day cycle), vincristine (1.4 mg/m^2), and dexamethasone was administered. Again, she did not tolerate this chemotherapy, and no additional systemic chemotherapy was offered. A repeat bone marrow aspirate showed that the pre-B cell immature lymphocytes persisted. Hypothesizing that a portion of her pancytopenia was due to the underlying MDS, she received AZA as per protocol for MDS (75 mg/m^2, subcutaneously for seven consecutive days out of a 28 day cycle) with the objective of improving cell counts and decreasing transfusion requirements. *Results.* She received a total of 6 cycles of AZA, with a significant hematological response. There was improvement in all of her cell lines, with a platelet count rising from 26 at presentation to 65 after four cycles of azacytidine. A repeat bone marrow aspirate performed after four cycles of AZA showed the immature B-cell population, which had represented 43% of all nucleated cells initially, had been reduced to 2% while the del20q abnormality remained. *Conclusion.* AZA has demonstrated activity in patients with MDS and in AML, but not in ALL or other lymphoproliferative disorders. In this ALL patient with underlying MDS, AZA resulted in significant anti-leukemic activity. Azacytidine deserves further laboratory and clinical investigation as a possible novel agent in the treatment of ALL.

Table. Bone marrow aspirate findings.

	WBC (10^9/L)	ANC (10^9/L)	Hb (g/L)	Platelet Count (10^9/L)	Blast Count (% of nucleated cells by flow cytometry)	Karyotype
April 7, 2005 (Initial Presentation)						
	3.7	1.5	128	70	None	N/A
January 23rd, 2009 (Clinical Deterioration)						
	2.7	1.35	106	26	43%	del20q
April 24, 2009 (Post Systemic Chemotherapy)						
	2.8	1.29	86	24	28%	del20q
November 9th, 2009 (Post 4 Cycles 5-AZA)						
	3.52	1.58	102	64	2%	del20q

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FEASIBILITY OF HYPER-CVAD IN ADOLESCENTS AND ADULTS PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. Despite improvement in cancer treatment during the last decade, the majority of adult patients with acute lymphoblastic leukemia eventually relapses and has a long term survival of less than 40%. *Aims.* The objectives of the current study were to evaluate the rate of complete remission and long term survival of patients treated in a single institution with the Hyper-CVAD regimen (four cycles of fractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone alternating with four cycles of methotrexate and cytarabine). *Methods.* Between December 2000 and April 2009, 38 adolescents and adults patients admitted to our institution received at least one cycle of frontline Hyper-CVAD. No exclusions were made because of older age, poor PS, organ dysfunction, or infection (including HIV infection). *Results.* The median age of the patients was 27 years (range 13-71 years) and 14 (37.8%) patients were classified as standard risk according to MRC UKALL/ECOG criteria. We identified 10 patients with T immunophenotype (27%), 4 patients with Ph^+ (23.5% of patients where the research was performed) and one patient with HIV. A complete response (CR) was achieved in 36 patients (94.7%) and 2 patients (5.3%) died during remission induction. Only two patients received allogeneic marrow transplantation in first CR. After a median follow-up time for alive patients of 45.4 months (range 10 to 102 months), 16 patients (42.1%) are still alive, 13 in first complete remission. The median overall survival (OS) was 24.4 months and the estimated 5-year OS was 34%. Among patients that achieved CR, the median event (relapse or death) free survival (EFS) was 19 months and the 5-year EFS was 30.5%. The median OS (36.2 months) and EFS (25.5 months) were favorable for standard risk patients but the difference did not reach statistical signifficance. *Conclusion.* The Hyper-CVAD regimen yielded satisfactory results in our institution. The rates of CR and survival were similar to obtained by other published series.

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IMPROVEMENT IN SURVIVAL OF ADULTS WITH ACUTE MYELOID LEUKEMIA IN A BRAZILIAN UNIVERSITY CENTER FROM 1980S TO THE EARLY 21ST CENTURY

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Background. Treatment of acute myeloid leukemia (AML) has improved during the last decades with enhancement of induction and postremission chemotherapies, development of autologous and allogeneic transplantation and supportive care progress. Nevertheless, population-based studies describe lower rates of long-term remissions. Previous report from our group showed that 37.6% of patients were too ill to receive chemotherapy and that was a main cause of failure. *Aims.* To evaluate recent treatment outcomes of AML diagnosed patients admitted to the University Hospital - Federal University of Rio de Janeiro over the past 30 years. *Methods.* By chart review, we retrospectively evaluated all patients diagnosed with AML at our institution, from January 1979 to December 2008. The diagnostic of AML was performed based on available procedures at the time, including bone marrow aspiration, bone marrow biopsy, cytogenetic and immunophenotyping analysis. Cases were classified according to de French-American-British (FAB) criteria and cases of acute promyelocytic leukemia (M3) were excluded. Patients were arranged into curative-intent, paliative-intent or no chemotherapy groups. *Results.* Of 295 patients with AML identified in hospital records, 68 were excluded (65 APL and 3 previously treated in other institutions). Among 227 patients analyzed, 125 were male and 102 female and the median age was 45 years (12-91), with 24.7% older than 60 years. Remission induction chemotherapy was used in 169 patients and 52.1% reached complete remission and 36.1% died before remission evaluation. Overall survival (OS) at 3 and 5 years for all patients was 14% and 13%, and in the treated group 18% and 17%, respectively. The distribution of cases for each time period was: 39% in 1980s, 32% in 1990s and 29% in 2000s. Stratifying by decades, the median survival were 40, 77 and 112 days (P=0.01), and 5 year OS were 7%, 13% and 22%, respectively. The proportion of treated patients was respectively 66.3%, 75.3%and 84.6%. Among treated patients (n=169) the median survival were 68, 205 and 208 days (P=0.05), and 5 year OS were 10%, 17% and 26% in each decade, respectively. The decade distribution was associated with better survival in a multivariate analysis. *Conclusion.* Previously, our group reported that no less than 37% of patients with AML were too ill to receive any treatment. This is in agreement with the current data of 66.3% of treated patients during the 80´s. In the last decade almost 85% of patients were treated. The survival expectations of patients with AML have substantially improved during a 30 years period and seems to be closer to those reported for patients living in developed countries and treated in randomized clinical trials

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THE OUTCOME OF THE ACUTE LYMPHOBLASTIC LEUKEMIA PATIENTS TREATED WITH INTENSIVE CHEMO-RADIOTHERAPY FOR THE FIRST 45 DAYS OF DISEASE - ROMANIAN WORKING GROUP FOR ADULT ACUTE LEUKEMIA STUDY EXPERIENCE

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Background. The treatment is the main prognostic factor in adult acute lymphoblastic leukemia. The outcome of these patients is considerably determined by the first months of treatment. Minimal residual disease (MRD) negative patients after inductions have a better outcome *vs.* the MRD positive patients. *Material and Methods.* We have studied 42 adult patients with acute leukemia enrolled after written consent signature in Romanian National Protocol for acute lymphoblastic leukemia treatment (RWGALS-NP1) between 2007 and 2009. The range of age was 37.2 years (17-74). There were 5 bifenotypic acute leukemia, 1 T-ALL, and 5 Ph+ ALL. For first 45 days of treatment the patients received 2 inductions chemotherapy: Induction I: Dexametazone 10 mg/sm p.o. days 6-7, 13 - 16, Vincristin 2 mg i.v, days 6, 13, 20, Daunorubicine 45 mg/sm i.v. days 6-7 and 13 - 14, E.Coli Asparaginaze 5000 U/sm days 18, 20, 22, 24, 26. Induction II: Ciclofosfamida 1000 mg/sm days 26 and 46, AraC 75 mg/sm i.v. days 28 - 31, 35 - 38, and 42-45, Purinethol 60 mg/sm p.o days 26-46. For CNS prophylaxis the patients received: Metotrexat 15 mg i.t days 1, 28, 35, and 42 and radiotherapy 24 Gy, from day 24 until day 44. *Results.* After first induction (day 26), 25 patients (60%) were in complete remission and 7 patients (17%) in partial remission. Two patients died during first induction and other 2 patients refused to continue the treatment due to severe treatment-related toxicity (mucositis grade III-IV, liver toxicity and infections). More 8 patients had complete remission after the second induction (day 45). Overall complete remission day 45 was 78.5%. During the second induction (combined chemotherapy and radiotherapy), 82% of patients had severe treatment related toxicity (mucositis grade III-IV, liver toxicity, infections, thrombocytopenia with cerebral or gastric bleeding). For 35% of patients there were protocol violation concerning the doses and the timing of chemotherapy, with a range delay of 14 days. Three patients died during second induction and other 4 patients refused to continue intensive chemotherapy after second induction due to severe treatment related toxicity. Overall survival was 19 months and disease-free survival was 17 months. *Conclusion.* The intensive induction therapy for adult acute leukemia patients combining chemotherapy and radiotherapy is not feasible in low income countries due to the difficulties to provide the supportive therapy. The new protocols for treatment of acute lymphoblastic leukemia patients that avoid radiotherapy using liposomal cytarabine based CNS prophylaxis seem be more appropriate in low income countries.

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TREATMENT OUTCOMES OF ADULT ACUTE LYMPHOBLASTIC LEUKEMIA TREATED WITH CALGB 1188 WITHOUT L-ASPARAGINASE

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Background. Acute lymphoblastic leukemia (ALL) is a treatable hematologic malignancy .Importance of L-asparaginase in treatment of adult ALL is controversial. CALGB 1188 is an effective protocol for treatment of adult ALL. This protocol contains L-asparaginase with signifi-

cant side effects in adults. To find the importance of L-asparaginase , we evaluated patients treated with CALGB 1188 without L-asparaginase . *Materials and Methods.* we searched the hospital documents from 2007 to 2009 for ALL patients treated with CALGB 1188 but did not receive L-asparaginase . Response rate , disease free survival and overall survival of these patients were analyzed. *Results.* Seventeen patients were treated with above protocol . Patients' age ranged from 16 to 64 years with mean age of 24.8 years. 57% were male and 23% were female. Complete response was achieved in 65% (11 of 17 patients). Disease free survival was 11.8 months in patients with complete response. Median of overall survival was 14.3 months. 5 of 17 patients were alive at the time of writing this paper. *Conclusion.* In our study 65% of patients achieved CR that is acceptable in comparison with most other studies (64% to 74%), but it is lower than CALGB 1188(85%). DFS and OS were also less than CALGB 1188. The cause of this lower result can be omitting L-asparaginase. Other factors such as our ward's conditions, facilities, prognostic and cytogenetic factors have effects on CR and survival. Since this study was retrospective, we could not evaluate the exact roles of these factors. It seems that L-asparaginase is important in CALGB1188 and if possible it should not be omitted.

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PHILADELPHIA POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA. IMPROVED OUTCOME IN THE TYROSINE KINASE INHIBITOR ERA

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Background. The prognosis of Philadelphia chromosome (Ph) positive acute lymphoblastic leukemia (ALL) is poor. But the incorporation into frontline treatment of tyrosine kinase inhibitor (TKI) imatinib has significantly improved the antileukemic efficacy of induction therapy and the prognosis in these patients. *Aim.* To assess the prevalence of Ph^+ ALL in a cohort of Romanian ALL patients and their outcome after treatment with tyrosine kinase inhibitors and chemotherapy. *Methods.* There were 35 ALL patients diagnosed and treated in our institution between 2007 and 2009. There were 20 males (57%) and 15 females (43%), with median age of 38 years (19-66). Fifteen patients were diagnosed with preB/common ALL, 7 patients with mature B cell ALL, 11 patients with T cell ALL and the phenotype could not be determined in 2 patients. Eight patients (22%) presented Ph positive ALL. Four patients presented complex karyotype (in one case associated with Ph chromosome), there was a duplication of Ph chromosome in 1 patient and a loss of chromosome 9 in 1 patient. Among the Ph positive ALL patients the major and minor breakpoint cluster region (BCR) rearrangements were equally distributed. 24 patients presented with leucocytes<30000/μL and 11 patients with >30000 leucocytes/μl. There were 3 patients with clinical CNS disease and 2 other patients with blastic meningitis with no clinical expression. All patients were treated with ALL-type combination chemotherapy. Imatinib at 600mg/day was added to chemotherapy in 6 out of 8 Ph positive ALL patients. Allogeneic stem cell transplantation was only available in one patient, Ph-negative. *Results.* We observed 25 complete hematological remissions (71%). Of the 8 Ph positive ALL patients, complete response was obtained in 6 patients (75%); in 1 patient complete cytogenetic response was obtained and in another patient major molecular response was obtained. There were 11 relapses (44%) among the whole group, only one in the Ph positive group (16.6%). There were 3 deaths among the Ph+ALL patients, 2 of these patients dying soon after the diagnosis (before TKI treatment was started) and the third died after relapse and failure of salvage treatment with chemotherapy and second generation TKI nilotinib and dasatinib. The median overall survival (OS) (determined by Kaplan Meyer) for our cohort was 36 months and the median disease free survival (DFS) was 24 months. The median OS for Ph positive ALL patients was not reached, 53% being still alive as of February 2010. The median OS for non-Ph positive ALL patients was 36 months. The median DFS for Ph^+ALL patients was not reached, 53% being in remission while the median DFS for non-Ph positive ALL patients was 26 months. Despite this apparently better results in Ph-positive patients, the differences were not statistically significant. *Conclusions.* Even though Ph-positive ALL is considered to have a particularly poor prognosis, this did not reflect in our series, although the number of patients was small and the follow-up was relatively short. Is is possible that the routine introduction of TKI treatment in Ph-positive ALL may also improve the long-term prognosis of Ph positive ALL patients, even in the absence of allogeneic hematopoietic stem cell transplantation.

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ACUTE LYMPHOBLASTIC LEUKEMIA AND PREGNANCY: A CASE REPORT

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In August 2008 a 19 years old girl was referred to our institution because of superficial lymph nodes without systemic symptoms. The peripheral blood showed leucocytosis, anemia and thrombocytopenia (WBC 21800/mmc, Hb 9.9 g/dL, PLT 99000/mmc). The patient was at the 16th week of pregnancy. A lymph node biopsy was compatible with pheripheral T cell Lymphoma, while the bone marrow analysis by morphology and multiparametric flow cytometry were compatible with diagnosis of T Acute Lymphoblastic Leukemia ($cyCD3^+$, $cyTdT^+$, $cy79a^+$, $CD7^+$, $CD5^+$, $CD4^+$, $CD2^+$, $CD10^+$) with blast counts superior to 75%; the cytogenetic analysis showed a complex cariotype: 46,XX, add(2)(q?33), del(6)(q?31), del(10)(q?24). Our aim was to cure leukaemia possibly avoiding any hindrance against pregnancy. After informed consent the patient received induction chemotherapy with Adriamycin (30 mg/sqm iv) plus Oncovin (1.4 mg/sqm iv) days 8,15,22,29 and Prednisone 60 mg/sqm/d for 30 consecutive days. Prophylaxis on CNS was performed with intrathecal administration of liposomal Cytarabine days 1,15,30,45. A bone marrow biopsy performed at the fourth week of therapy showed a CR either morphological either at flow cytometry, the cariotype was normal too. The pregnancy was uneventful and the child was growing correctly. The second course of induction therapy consisted of Cytarabine (75 mg/sqm/d iv for 4 days/weekly for 4 cycles). At the 34th week of pregnancy an healty newborn baby was delivered by caesarum partum with a weight of 2100 g. After one month the patient received consolidation therapy with HAM regimen and collection of pheripheral blood stem cells. In Febrary 2009 she underwent autologous stem cell transplantation. Conditioning regimen consisted of Thiotepa (10 mg/Kg iv day -5) plus Cyclophosphamide (60 mg/Kg/d iv from day -3 to -2) followed by reinfusion PBSC for 9.75×10^6/Kg $CD34^+$ cells. She achieved haematological recovery at day +10. The extrahaematological toxicity during phase of cytopenia was mild and transient. In April 2009 she started maintance therapy according scheduled ALL GIMEMA protocol. The patient is still in CR and her little baby grows normally without apparent deformities. In conclusion the cure of ALL during pregnancy is possible safeguarding the foetus and its maturation. A reasonable therapeutic strategy should be warranted in order to avoid main problems due to single chemotherapy agents.

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CA 125 IS A USEFUL MARKER FOR SEROSAL INVOLVEMENT IN CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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*Background.*Cancer antigen 125 is a glycoprotein expressed in normal tissues originally derived from celomic epithelia and has long been known as an ovarian tumor marker, but more recently, it has been known that its serum level is elevated in various benign and malignant conditions.It has also been reported to be elevated in adults with acute leukemia with extramedullary localization and serosal effusion. *Aims.* We aimed to study CA125 as a marker for detection of serosal involvement in children with acute lymphoblastic leukemia(ALL). *Methods.* Thirty patients with ALL,21 males and 9 females, their age ranged from 1.5 to 13 years and 20 healthy controls were included.Serum level of CA 125 was measured at initial diagnosis and after remission in the patient group while it was measured once in the control group. *Results.* Serum level of CA 125 was significantly higher in our patients than the control group and it was significantly lowered after achievement of remission. The highest levels were observed in patients with serosal involvement. *Summary/Conclusions.* CA 125 is a useful marker for serosal involvement in children with ALL.

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SUCCESSFUL TREATMENT OF L-ASPARAGINASE INDUCED PANCREATITIS WITH OCTREOTIDE IN A CHILD WITH ACUTE LYMPHOBLASTIC LEUKEMIA

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Acute pancreatitis (AP) is one of the complications that occur during chemotherapy in acute lymphoblastic leukemia (ALL) patients. The main etiology of AP in ALL patients has been reported to be associated with L-asparaginase (L-asp) therapy. Somatostatin, a naturally occurring tetradecapeptide, and its synthetic analogue octreotide, have been used to treat various pancreatic disorders in adults. The pediatric experience with this medication is limited. We report a case of a 7 year-old female diagnosed with common ALL stratified to the high risk (HR) BFM95 protocol due to leukemic infiltration of the CNS and blasts >1000/_L on day 8. After the induction therapy and towards the end of the HR 1 sheet of the protocol after the administration of L-asp (25,000 IU/m^2) she developed abdominal pain with bilious emesis, and watery guaiac positive stools. Laboratory studies included normal serum electrolytes and renal function studies, hypertriglyceridemia and transaminasemia. Serum amylase was 787 IU/L (normal range: 28-100 IU/L), serum lipase was 2560 IU/L (normal range: 7-60 IU/L) and urine amylase 6550 IU/L(normal range: 0-460 IU/L). Ultrasonography showed changes indicative of acute pancreatitis. Initial management included nasogastric tube placement, bowel rest, analgesics, and broad spectrum antibiotics. Octreotide therapy was initiated within the first 12 hours for a total of 7 days. In the following 5 days her clinical status showed steady improvement as evidenced by decreasing levels of serum and urine amylase to 85 IU/L and 61 IU/L, respectively. After recovery from acute pancreatitis the patient was not rechallenged with L-asp However additional medications that have been associated with pancreatitis, including corticosteroids, 6-mercaptopurine and trimethoprim-sulfamethoxazole were re-instituted following resolution of the acute episode, without recurrence of pancreatitis and were thus not thought to be the major contributor to pancreatic toxicity. Clinicians should be aware of AP in patients presenting with acute gastrointestinal symptoms, especially in those being treated with a high-risk protocol. A high index of clinical suspicion will allow early diagnosis and appropriate therapy that may improve the outcome. Octreotide as part of the management of L-asp induced pancreatitis in children is safe and effective in limiting the severity of the disease process.

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PROLIFERATION OF ACUTE MYELOID LEUKEMIC CELLS MEASSURED BY KI-67 AND PCNA EXPRESSION

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Autoradiographic studies in 80's revealed that myeloid leukemia cells have different proliferation rate, and in general it is low, with most cells in G0 phase of cell cycle. Introduction of flow cytometry with DNA/RNA measurement confirmed those data. As these measurements have certain technical obstacles, proliferative markers were introduced in several human neoplasm to evaluate cell proliferation and therefore behaviour of tumour. *Objective.* We analyzed proliferative fraction of leukemic cells by the application of monoclonal antibodies to Ki-67 and PCNA proliferative markers, and compared these findings with clinical features and initial treatment outcome (CR/NR). *Patients and Methods.* In order to avoid permeabilization of cells for nuclear antigens, we analyzed proliferation on bone marrow samples (trephines) in a cohort of 42 patients suffering from *de novo* AML. Mean age was 46 yrs. (19-66 yrs), and all were treated with similar treatment (ADE/MAE 30 pts, and DA/MC schedule 12 pts). After obtaining informed consent, bone marrow biopsy samples were taken at diagnosis and fixed in B5, embedded in paraffin. After deparaffinization, antigen retrieval and immunohistochemistry was performed by use of commercial Ki-67 (MIB-1) and PCNA (PC-10) antibodies and imaging kits (LSAB2, Dako Danmark) according to manufacturer prescription. Statistical analysis included parametric and nonparametric tests. *Results.* According to FAB classification 3 patients had M1 and 18 M2. Seventeen patients had AML M4 and 3 patients M5. One patient had AML M0. Fifteen patients had trilineage dysplasia. Twenty eight patients achieved CR (67%) and 14 were non responders. Mean Ki-67 positivity was 9.05±17.7% (0-82%). In 15/42 patients (35.7%) Ki-67 was absent, and in 38.1% was <10% of leukemic cells. In 11/42 patients (26.2%) Ki-67 was positive in >10% of leukemic cells. Mean PCNA positivity was higher than Ki-67, 34.7±25.1% (0-90%). In only 4/42 patients (9.5%) PCNA was absent and in other 4 (9.5%) was <10%. In 13/42 patients (30.9%) PCNA was positive in >40% of leukemic cells. There was good linear and rank correlation between these two proliferation markers (P<0.05). Patients with trilineage dysplasia had higher Ki-67 positivity (14 *vs.* 6.3%, MWU test P<0.05) but not PCNA positivity. There were no correlation between percentage of Ki-67 or PCNA positive leukemic cells according to morphology type or cytogenetic features. Patients responded to induction chemotherapy (achieved CR) had significantly lower proliferation measured through Ki-67 positivity (CR 4.6 *vs.* 17.9% for NR, MWU P<0.05), but not by PCNA determination (31.2 *vs.* 41%, P>0.05). Patients with lower proliferation, Ki-67 and PCNA labeled leukemic cells <10%, had better survival than others (log rank test for Ki-67 P=0.06, for PCNA P>0.05). *Conclusion.* Our results in a small cohort of patients as pilot trial confirmed that leukemic cells in AML are dormant, in most cases are non proliferative, which is similar to other findings. There is a good correlation between Ki-67 and PCNA proliferation markers. Also, patients with lower proliferation have better treatment outcome (CR achievement) and better survival.

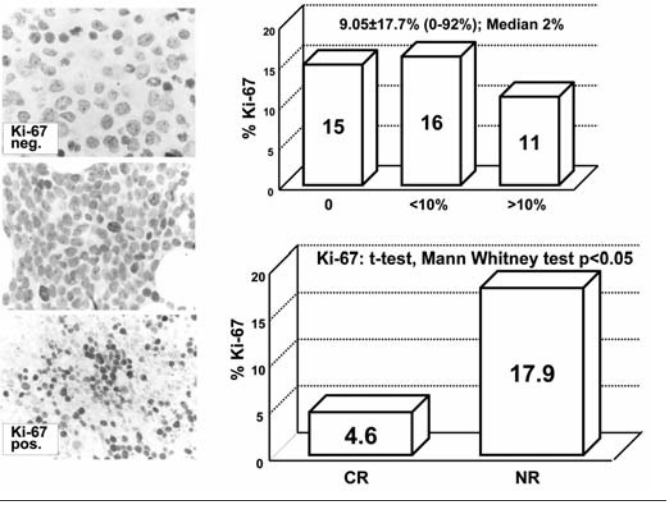

Figure. Proliferation measured by ki-67 and outcome.

1201

MRD MONITORING IN ADULT AML WITH NORMAL KARYOTYPE. COMPARAISON OF WT1 VERSUS NPM1M WHEN BOTH MARKERS ARE PRESENT.

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Background. Minimal residual disease (MRD) monitoring has been proved to be useful to guide therapeutic strategies in adult AML. The applicability of MRD monitoring is limited by the presence of a suitable RT-PCR detectable genetic marker. Thus, the over expression of Wilms' tumour gene (Wt1), a transcription factor implicated in neoplasm proliferation, in the majority of adult AML, make it suitable as a commonly used MRD marker. However, Wt1 is expressed at a low level in normal blood and marrow undifferentiated cells. Thus, should we replace the use of more specific markers like mutated NPM1 (NPM1m) by Wt1? *Patients and Methods.* We chose 25 adult AML patients, treated into or according to French multicentre clinical trials (ALFA and GFM) whose molecular screening at diagnosis found a WT1 over expression and a NPM1 mutation. They were 11 males and 14 females, median age 56 years (range: 37 to 71). These patients had a MRD monitoring using in parallel both markers. Quantification of Wt1 and mutated NPM1 was performed according to the ELN recommendations using primers and probes already published (respectively van Dijk, 2002 and Falini, 2005). *Results.* We analysed 143 samples (119

blood samples, 21 bone marrow samples and 3 CSP samples). Quantification was performed at diagnosis and on MRD follow up samples using both markers. On diagnosis samples, there was no correlation between NPM1m expression and Wt1 expression. In most cases (12/25), WT1 was >10% and NPM1m was >10%. In 8/25 cases, WT1 was <10% and NPM1m was >10%. In 4/24 cases, Wt1 was <10% and NPM1m was < 10%. In 1/25 case, Wt1 was >10% and NPM1m was <10%. On MRD samples, however, 91/118 (77%) MRD samples were concordant in term of negativity or positivity of Wt1 and NPM1m expression. 17 /118 (14%) were positive with both markers. 74/118 (63%) were negative for both markers. However, 27/118 (23%) samples were discordant. In 3/118 (2.5%) samples, W1 was considered to be slightly over expressed and NPM1m expression was not detected. But in 24/118 (20%) samples, NPM1m was detected and WT1 was below the threshold of residual positivity (0.045% in blood and 0.28% in bone marrow) observed in normal controls (mean + 2SD). In all these cases, this lack of sensitivity was responsible for either a wrong perception of the decrease of the tumour burden (14/24, 58%), or a delayed haematological relapse anticipation (10/24, 42%). In 6/8 cases of relapse, a slow tumour burden clearance was associated with a relapse and in all these cases, this relapse could have be predicted several months earlier using NPM1m quantification but not using Wt1 quantification. *Conclusion.* Wt1 over expression is a useful marker for MRD detection. However, the presence of a Wt1 normal hematopoietic cell low level of expression decreased its sensitivity and is responsible for a loss of information on the kinetic of the tumour burden evolution. Therefore, if there is a choice, it seems preferable to use a more specific marker like NPM1m.

1202

WITHDRAWN BY AUTHOR

1203

LOW STRUCTURAL MAINTENANCE OF CHROMOSOMES 1A PROTEIN EXPRESSION AT DIAGNOSIS PREDICTS POOR PROGNOSIS IN ACUTE MYELOID LEUKEMIA

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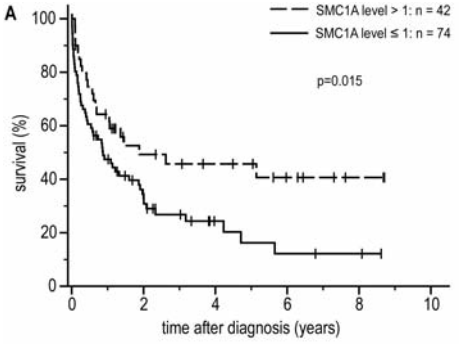
Background. Acute myelogenous leukemia is a genetically heterogenous disease with several risk factors implicating a poor prognosis. One of the most important independent risk factor in AML is the age at diagnosis. Older patients with AML generally have a poor prognosis which suggests that the biology of AML in elderly patients differs from AML in younger patients. Little is known about the differential gene expression profile of leukemic cells in elderly *vs.* younger AML patients. *Aims.* The aim of the study was to identify age-related genes in AML and evaluate the impact of the protein expression level of these genes on the prognosis of AML patients. *Methods.* Gene expression profiling was carried out in bone marrow mononuclear cells to identify age related changes in 67 AML patients of different age (range: 17 to 80 years, median: 61 years). Among the genes that correlated with age, SMC1A was selected for protein expression studies. A tissue array was generated containing bone marrow histology samples of 135 patients with newly diagnosed AML of different ages (range: 17 to 84 years, median: 61 years) and probed with an antibody against SMC1A. Protein expression was quantified by the DAKO scoring system.



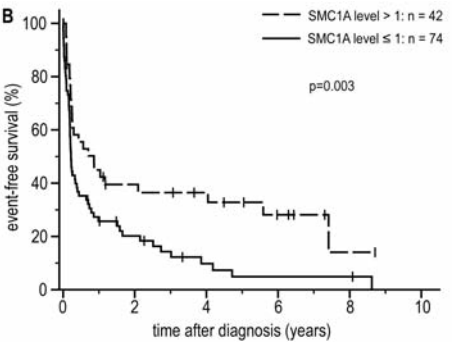


Figure.

Results. 131 genes showed a significant correlation between mRNA expression levels and patient age. Increasing age was associated with significantly decreased mRNA levels of SMC1A. 116 patient samples were evaluable for SMC1A protein expression, and expression of SMC1A protein was low or absent in 74 (64 %) out of 116 AML specimens. On the protein level, SMC1A expression did not correlate with patients' age at diagnosis. Both event free survival (2.6 months *vs.* 10.3 months, P=0.003, Figure) and overall survival (10.4 months *vs.* 22.6 months, P=0.015, Figure) were significantly worse in patients with low or absent SMC1A protein expression. In a multivariate analysis, SMC1A protein expression level remained a significant prognostic factor for event free survival (P=0.014) with a borderline significance for overall survival (P=0.066). *Summary/Conclusions.* We identified 131 genes with putative age-dependent microarray mRNA expression and identified low levels of SMC1A protein expression as a marker for poor prognosis in patients with newly diagnosed acute myeloid leukemia.

1204

SHORT WT1 VARIANT OF WILMS TUMOR GENE IS EXPRESSED IN LEUKEMIC CELL LINES, PATIENTS WITH AML, MDS AND IN CML BLAST CRISIS

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Prognostic significance of total WT1 expression has been proved both for AML and CML, however, the oncogenic effects of WT1 in leukemic cells have not been fully understood yet. In 2004 Dallosso *et al.*. described a short WT1 (sWT1) variant and in 2006 Hossain *et al.*. suggested a connection of sWT1 with leukemias. sWT1 as a part of total WT1 is transcribed from the alternative first exon 1a. Corresponding protein, AWT1 isoform is N-terminally truncated with the lack of transcriptional repression domain, which is supposed to be the cause of its oncogenic properties. To examine sWT1 as novel prognostic marker for leukemic patients and for further understanding the role of sWT1 in leukaemias we have analysed sWT1 and "full length"WT1 expression both on mRNA and protein level in AML, CML and MDS patients. Novel real-time PCR system was established for sWT1 and "full length"WT1 quantification. The two variants were quantified in two separated PCR reactions by combining two different forward primers (one localized on exon 1 and the other on exon 1a) with a common reverse primer and TaqMan probe using GUS as a control gene. Quantification utility was confirmed by high correlation (R^2=0.98) between the sum of sWT1 and full length WT1 with total WT1 measured by standard method according to Kreutzer *et al.,* 2001. Western blot analysis was performed using antibody against the C-terminal region of the WT1 protein. So far, total leukocytes of 41 AML, 5 MDS and 30 CML patient samples were analysed together with K562, CML and JURL cell lines and healthy donors (n=10). Transcript of sWT1 variant was found in K562, CML and JURL cell lines as well as in patient samples but mostly at low levels. We found that sWT1 variant was expressed in CML patients in blast crises but not in patients in chronic phase. Among different AML FAB subtypes, equal levels of sWT1 and WT1 were observed in AML M5b patient, however, sWT1 variant was undetectable in AML M5a patients. MDS patients showed large range of sWT1 expression. High level of full length WT1 was detected in all cell lines and patients samples except of CML patients with major molecular response (<0.1% BCR-ABL), where sWT1 was undetectable. Corresponding results were found at sWT1 and full length WT1 protein lev-

els. In conclusion, sWT1 is expressed at low levels in AML, CML and MDS patients. As sWT1 emerge in AML and in advanced phases of CML, but not in the chronic phase, sWT1 emergence seems to be related to blast cell phenotype. Expression studies of separated cell populations and leukemic progenitor cells are ongoing to examine that idea. Observed differences in sWT1 levels among AML patients tempts to hypothesize prognostic value of sWT1 levels for AML patients. Whether there is any such value and whether such low levels of sWT1 might be of any importance for leukemia pathogenesis remains to be examined. Supported by MZOUHKT2005 and NS 10488-3.

1205

7.1 ANTIBODY REACTS WITH LEUKEMIC CELLS OF ACUTE MYELOID LEUKEMIA BEARING INV(16)

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Background. Several antigenic patterns have been associated with genotypic aberrancies in acute leukemia. The expression of the human counterpart of mouse NG2 molecule, targeted by 7.1 monoclonal antibody (MoAb), has been demonstrated in association with rearrangement of the mixed lineage leukemia (MLL) gene in acute leukemia. *Aims.* The aim of the study was to investigate the expression of 7.1 MoAb in an unselected cohort of patients with acute myeloid leukemia (AML) in order to establish its correlation with specific genotypic groups. *Methods.* An extensive panel of quadruple combinations of MoAb was applied on a suspension of bone marrow or/and peripheral blood of AML samples at diagnosis. CD45 PerCP was applied in every tube to allow gating of cell populations. The expression of NG2 was assessed using the following staining: CD65-FITC/7.1-PE/CD45-PerCP/CD34-APC. MoAb 7.1 PE was purchased from Beckman-Coulter. 50,000 events per tube were acquired through a FACSCalibur flow cytometer. Analysis of antigen expression was performed through Infinicyt software. NG2 expression was explored on leukemic cells, gated on the basis of CD45 and side scatter, through: i) percentage of 7.1+ cells; ii) mean fluorescence intensity corrected for background fluorescence (MFI) determined by an isotype control; iii) coefficient of variation (CV) of fluorescence intensity. Cytogenetic aberrancies were determined by conventional karyotyping and by FISH for inv(16), t(8;21), t(15;17) and MLL; mutations of NPM1 gene were revealed by immunohistochemical detection of aberrant cytoplasmatic protein localization on trephine biopsy.

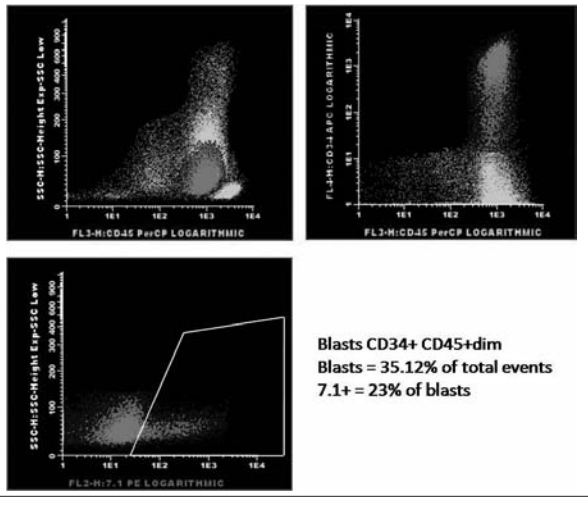

Figure. Expression in a case of AML with inv(16).

Results. Cytogenetics. 64 AML patients were studied. According to genotype, they were subdivided as follows: a) t(15;17) # 6; b) t(8;21) # 4; c) inv(16) # 5; d) normal karyotype # 28, of whom 16 NPM1-wild type and 12 NPM1-mutated. No cases with MLL rearrangements were seen. According to SWOG stratification, 10 and 7 patients had high- and intermediate-risk (other than normal) karyotype respectively. Conventional karyotyping was not possible in 4 cases due to lack of growth. Flow cytometry. Leukemic cells were identified by dim expression of CD45 and low scatter signal; overall, blasts represented a median of 50.78% of global cellularity. No expression of 7.1 was detectable in patients with t(15;17), t(8;21) or other cytogenetic aberrancies. Neither within normal karyotype group, regardless of NPM1 status, 7.1 resulted positive in any case. Of note, we detected a partial expression of 7.1 on blasts from AML with inv(16). Specifically, all 5 patients with such genotype showed 7.1+ cells, with a median percentage of 23% (range 10-28) within blasts. Median MFI was 4.91 (range 3.47-9.86); median CV 275.41 (257.62-291.89). Apart from inv16, expression of 7.1 was seen in an AML case diagnosed as blastic plasmacytic dendritic cell neoplasm according to WHO classification; this finding was not unexpected since Orfao *et al.* (Haematologica 2004) have reported it in this entity. *Summary/Conclusions.* Our study describes the correlation between the expression of 7.1, so far associated with MLL abnormalities, and AML with inv(16). If confirmed within a larger cohort, this phenotypic aberrancy should be deepened for potential prognostic role and minimal residual disease assessment.

1206

BAG-1 IN ACUTE LEUKEMIA: HUNDRED FACES OF A SINGLE PROTEIN

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Introduction. Bcl-2 associated athanogene-1 (Bag-1), a founding member of BAG protein family, is a multifunctional protein which has a role in a wide range of cellular processes including apoptosis, cell survival, transcription, cell motility and proliferation. The involvement of Bag-1 in different cellular pathways can be in part examined by the sub-cellular compartmentalisation of the three major Bag-1 isoforms (Bag-1L, Bag-1M and Bag-1S), generated by alternative translation from a single mRNA, and in part by its interaction with a large number of disparate proteins, including Bcl-2, Raf-1, nuclear hormone receptors, subunits of the ubiquitinylation proteasome complex, Hsc70 and Hsp70. *Aims.* The elevated level of Bag-1 protein has been confirmed as a considerable index in several malign diseases. To determine significance Bag-1 might have in the processes of leukemogenesis a sequence of human leukemic cell lines and pediatric bone marrow samples with confirmed acute myeloid leukemia were included in our research. Our goal was to clarify the molecular mechanisms potentially in charge for Bag-1 action, in either leukemic cell lines or primary cell cultures. *Methods. In vitro* studies were based on a small-interfering RNA (siRNA) approach and the results were validated using standard techniques for mRNA and protein expression study. Assays for cell cycle and apoptosis detection were performed. *Results.* The protein study revealed elevated Bag-1 levels in human leukemic cell lines of both myeloid (ML2, THP1, NOMO1, NB4, MV4;11 and HL60) and lymphoid (REH, RS4;11, 697 and JURKAT) origin. A different expression pattern of Bag-1 protein isoforms was noted for two considered groups of patients, AML or ALL, with changes in protein expression profile at different point of the disease. After Bag-1 was knockdown, a modest effect on cell death or cell cycle profile was observed for the human cell lines while primary cultures showed to be more sensitive to Bag-1 silencing. However, significant decrease was confirmed at the expression level of a wide range of proteins, specially the ones involved in the regulation of apoptosis (Bcl-2, PARP, Caspase-3), cell cycle (p27, CDK2, Cyclin D1) and autophagy (LC3, p62), without affecting the mRNA levels. When double silencing experiments of Bag-1 and Bag-3 (a family co-member) were performed, the effect on cell death and cell cycle arrest were found enforced, suggesting a connection between two proteins to be significant for cells faith in leukemia. *Conclusions.* Results indicate that the role of Bag-1 in cell death prevention might be more related to lymphoid leukemia, while it might be considered more significant for cell differentiation in myeloid cells. At the same time, elevated Bag-1 protein expression levels in acute leukemia indicates its possible significance for AML and ALL growth. A different expression profile of Bag-1L, Bag-1M and Bag-1S isoforms in myeloid with respect to lymphoid leukemia could lead to the hypothesis that Bag-1 might play a role in leukemia switch, triggering to either ALL or AML phenotype.

1207

PRIMARY HUMAN AML CELLS CAN EXPRESS CALRETICULIN ON THE CELL SURFACE AND RELEASE HEAT SHOCK PROTEIN 70 (HSP70) AND HSP90 DURING APOPTOSIS

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Background. Experimental studies strongly suggest that induction of immunogenic apoptosis with exposure of calreticulin on the cancer cell surface is important for induction of anticancer T cell responses. The calreticulin exposure seems essential for uptake and presentation of cancer-associated antigens by dendritic cells. Immunogenic apoptosis is in addition characterized by plasma membrane expression and release of

Heat shock protein (HSP) 70 and HSP90, and these molecules favor cross-presentation of cancer-derived antigens by dendritic cells. Finally, chemotherapeutic drugs seem to differ in their ability to induce immunogenic apoptosis; experimental studies suggest that this phenotype can be induced by anthracyclines but not mitomycin. *Aims.* The aim of the study was to investigate whether induction of apoptosis in primary human AML cells is associated with an immunogenic phenotype including calreticulin exposure and HSP70/HSP90 release. *Methods.* Primary human AML cells derived from 15 consecutive patients were cryopreserved and later thawed and cultured under highly standardized *in vitro* conditions. We used a highly standardized experimental model, and the spontaneous/stress-induced *in vitro* apoptosis that is observed during culture has been characterized in detail previously (Ryningen A, Leuk Res. 2006; 30: 1531-40). Cells were cultured in medium alone and in the presence of daunorubicin, cytarabine, mitomycin, all-trans retinoic acid (ATRA) or valproic acid. Except for mitomycin all these drugs are used in the treatment of AML. AML cell viability and exposure of calreticulin were determined by flow cytometry and HSP70/HSP90 release by ELISA analyses. *Results.* All three cytotoxic drugs caused an extensive *in vitro* apoptosis after 18 hours of incubation. We therefore investigated calreticulin exposure after 4 hours of *in vitro* culture when AML cells generally showed a viability >70% and at the same time an increasing population of dead cells compared with shorter incubations. A dye exclusion assay was used to discriminate between live and dead cells, and these two subsets were analyzed separately with regard to calreticulin exposure. We first investigated stress-induced *in vitro* apoptosis for cells cultured in medium alone, and a wide variation was observed between patients especially for the dead/dye-permeable cell subset (Figure 1). This variation was also detected after exposure to all three cytotoxic drugs, ATRA and valproic acid. Detectable release of HSP70 and especially HSP90 was observed during stress-induced apoptosis and after exposure to cytotoxic drugs, but again a wide variation between patients was observed. Finally, primary AML cells were derived before and during *in vivo* treatment with ATRA plus valproic acid for patients included in a clinical study, and the wide variation in calreticulin exposure and HSP release was maintained during this treatment. *Summary/Conclusions.* Primary human AML cells show calreticulin exposure and release HSP70 and HSP90 during apoptotic cell death. There is a wide variation between patients both with regard to calreticulin exposure and HSP release during spontaneous/stress-induced *in vitro* apoptosis, and this variation is maintained even in the presence of daunorubicin, cytarabine, mitomycin, ATRA and valproic acid. Thus, induction of immunogenic apoptosis in primary human AML cells is strongly influenced by patient-specific biological differences.

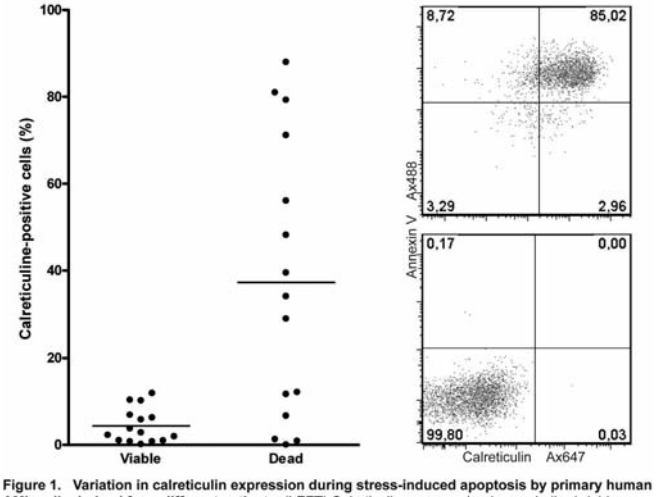

Figure 1. Variation in calreticulin expression during stress-induced apoptosis by primary human AML-cells derived from different patients. (LEFT) Calreticulin-exposure by dye-excluding/ viable and dye-permeable/ dead AML cells derived from 15 patients. (RIGHT) Exposure of calreticulin (x-axis) and Annexin-V (y-axis) by dye-permeable/ dead AML cells derived from two different patients.

1208

INHIBITION OF CELL CYCLE PROGRESSION IN T CELLS AS IMMUNE ESCAPE MECHANISM IN AML

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Background and Aims. Although leukemic blasts express immunogenic tumor-associated antigens, specific T cell responses tend to be severely impaired in acute myeloid leukemia (AML) patients. While contact dependent anergy induction resulting from a lack of costimulation has been previously described, the role of soluble immunosuppressive factors in this disease is less well understood. Here, we analyzed cellular und molecular effects of cell contact independent immune escape mechanisms in AML. *Methods.* $CD4^+$ T cells from healthy donors were cultured in 70% conditioned medium obtained from HL-60 cell cultures supplemented with 30% fresh RPMI medium. PMA and ionomycin were added for mitogenic stimulation. The induced proliferation was evaluated by propidium iodide staining and FACS-based cell cycle analysis was performed. Conditioned medium from a carcinoma cell line (LoVo) was used as a control in subsequent experiments. Expression of positive (i.e., Cdk4, Cyclin E, Cyclin A) and negative (p27Kip1) regulators of cell cycle progression was assessed by western blot analysis. *Results.* Our studies demonstrate that $CD4^+$ T cells cultured in HL-60 conditioned medium show profound alterations in cell cycle regulation with a predominant arrest in G1 phase. Western Blot analyses revealed considerable downregulation of molecules required for G1-S-transition (i.e., Cdk4, Cyclin E, Cyclin A) while no differential regulation was found for Cdk inhibitor p27Kip1. *Conclusion.* AML blasts can induce growth arrest in activated T cells in a cell contact independent manner. The mechanism demonstrated here might contribute to an ineffective antileukemic immunity *in vivo*. While the responsible soluble factors remain to be identified, this study points towards cell cycle regulators as a potential novel target for the optimization immunotherapeutic approaches.

1209

PROMYELOCYTIC LEUKEMIA: MRD DETECTION AND ITS CORRELATION WITH CLINICAL OUTCOME

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Background and Objectives. Acute promyelocytic leukemia (APL) is a distinct subtype of acute leukemia characterized by a balanced reciprocal translocation between chromosome 15 and 17 resulting in the chimerical gene encoding PML-RARα protein. Our aim was to study the PML-RARα fusion gene by real-time RT-PCR before and after therapy in a trial to asses its prognostic value and its impact in monitoring MRD to improve therapeutic strategies and decision making. Patients and Method: The study included 41 newly diagnosed APL patients. In addition to the standard work up, cytogenetic and FISH testing were performed at diagnosis and at CR. RT-PCR was done before treatment and after consolidation. We calculated the levels of PML-RAR_ by different *Methods.*1-The number of transcripts. 2-The ratio % which is calculated as: Copy number of PML-RARα /copy number of ABL. 3-The normalized copy number (NCN) which is calculated as: Copy number of PML-RARA/ copy number of ABL x10000. To analyze the different prognostic factors, we correlate them to MRD cut-off values which were for the ratio% (1×10^{-3}), and (NCN=10) for NCN method. Accordingly patients were divided into: Low risk patients: those with ratio% $<10^{-3}$ or NCN<10 and high risk patients: those with ratio% ≥10-3 or NCN>10. Treatment plan: Induction and consolidation regimen included ATRA 45 mg/m^2 P.O. daily and Daunorubicin 60 mg/m^2 I.V. day 1-3 for three courses.Maintenance therapy was given with 6 mercaptopurine and methotrexate with ATRA intermittent courses for two years.Informed consent was signed by all cases. *Results.* Median age was 36 years (18-66) with females to male ratio of 1.2:1. Only 30 patients achieved hematologic remission and followed up for MRD detection while eight cases failed to achieve CR and three cases showed early death.The decrease in expression level of PML-RARα after treatment was highly signficant. During the observation period of 39.1 months (1-56), six cases showed relapse. The time elapsing from the detection of molecular relapse till the appearance of hematological relapse ranged from 1-3 months with most cases clustering in the 3rd month (4patients). The cumulative disease free survival(DFS) was 69.4%. The overall survival(OS) was 76.6% with a mean of 49.85 months with 95%confidence interval of (44.8-54.8). We found significant longer DFS and OS for the low *vs.* high risk patients using the NCN cutoff at post consolidation analysis (P=<0.001 and 0.009 respectively). We found also high statistical significant correlation between log reduction of 2or more after consolidation and longer DFS but not OS. *Conclusions.* PML/RARα NCN method is simple and accurate; its use enables standarization and avoid differences among laboratories. Monitoring patients at short time points is valuable and essential for decision making and choice of proper therapy.

1210

PML/RARA BCR1 AND BCR3 TYPE APL PATIENTS HAVE A DIFFERENT PATTERN OF PRAME, HSP27, S100A4, P21 AND SURVIVIN GENE EXPRESSION

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Background. Most frequent cytogenetic hallmark of the acute promyelocytic leukemia (APL) is a translocation t(15;17) that results in the fusion chimeric oncogene PML/RARA. There are 3 different transcriptional variants of PML/RARA: bcr1, bcr2 and bcr3, and they depend on the molecular anatomy of genomic breakpoints. Bcr1 and bcr2 are very similar from the structural point of view. Frequency of bcr1 and bcr3 in adults are rather equal but in different populations there may be a little shift of the balance into the favor of one of them. The frequency of bcr2 in adults is rather low (about 5%). There are contradictory data about the influence of the PML/RARA transcriptional types on the clinical outcome of APL with a few reports showing that patients with bcr3 may be less favorable. We believe that a thorough elucidation and comparison of molecular events that depend on the presence of bcr1 or bcr3 PML/RARA would explain the possible difference in response to treatment in the corresponding APL patients. *Aim.* To study bcr1 and bcr3 PML/RARA positive primary APL patients in order to find a difference among them in expression pattern of genes that may possibly be involved in APL pathogenesis. In this purpose we decided to study an expression level of PRAME, Hsp27, S100A4, p21, WT1, XIAP, survivin and FLT3 genes. *Methods.* To perform this study we have used TaqMan RQ PCR (ABL gene as a housekeeping control gene) and non-parametric methods of statistical analysis of the data obtained. *Results.* We have not found any significant difference of WT1, FLT3 and XIAP gene expression in bcr1 (N=32) and bcr3 (N=45) PML/RARA positive primary patients. On the other hand in these groups of APL patients there was a statistically significant difference in PRAME, Hsp27, S100A4 and survivin gene expression (medians for PRAME(bcr1)=0,034 *vs.* PRAME(bcr3)=0,202, P=0,042; Hsp27(bcr1)=11,69 *vs.* Hsp27(bcr3)=6,42, P=0,0031; S100A4(bcr1)=4,33 *vs.* S100A4(bcr3)=8,86; survivin (bcr1)=1,67 *vs.* survivin(bcr3)=9,19, P=0,0027). *Conclusion.* Our data suggest that in the case of APL there are a number of genes expression of which may depend on the transcriptional type of PML/RARA oncogene. Elevated expression of PRAME, S100A4 and survivin and attenuated expression of Hsp27 in bcr3 cases of APL may be responsible for the less favorable outcome of this type of APL.

1211

ASSOCIATION OF HIGH-LEVEL EXPRESSION OF BCL2L12 GENE WITH ADVERSE OUTCOME IN PATIENTS WITH ACUTE MYELOID LEUKEMIA

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Background. It is well known that changes of the expression level of the main regulators of the apoptosis machinery, such as BCL2, BAD and BAX, contribute to poor outcome in patients with acute myeloid leukemia (AML). BCL2L12 is a novel member of the BCL2 family. Analysis of BCL2L12 mRNA expression in breast and colon cancer, have revealed its involvement in apoptosis in malignancies. However, its role in AML has not been studied yet. *Aims.* In the present study, we investigated the expression profile of the novel apoptosis related gene, BCL2L12, in blood samples of 46 patients with *de novo* AML and 21 samples of healthy controls. We correlated these data with clinical and pathological characteristics, in order to examine potential use of *BCL2L12* mRNA expression as a novel molecular marker in AML. *Methods.* Total RNA was extracted from mononuclear cells and reverse-transcribed into cDNA. We used a highly sensitive semiquantitative real time PCR method based on SYBR Green I chemistry. The internal control gene was *GAPDH.* Relative quantification analysis was performed using delta-delta Ct method. The association of the *BCL2L12* mRNA expression level with clinical and pathological parameters of the disease was evaluated by using chi-square test or the Fisher's exact test, where appropriate. *Results.* In this study the median expression level of *BCL2L12* in 46 *de novo* AML patients was 5.75 while it was 14.82 in 21 healthy individuals. However, this difference was not statistically significant (P=0.14). An optimal cut off value for *BCL2L12* expression of 3.42, equal to the 3rd quartile, was applied to discriminate BCL2L12-positive from BCL2L12-negative cases. Statistically significant relationship was found between *BCL2L12* expression level and CD117 expression (P=0.032), the presence of splenomegaly (P=0.044) and chemotherapy response (P=0.034). Most importantly, we evaluated *BCL2L12* gene expression as a predictor of survival in AML patients. According to univariate data analysis, leukemia patients with positive *BCL2L12* expression status were 3.42 times more likely to relapse (P=0.004) and 3.14 times more likely to die (P=0.007) than patients with negative *BCL2L12* expression status. In multivariate analysis, *BCL2L12* expression was found to be significant prognostic marker for DFS (P=0.014). Kaplan-Meier survival curves demonstrated that BCL2L12-negative patients had substantially longer DFS and OS (P=0.0015 and P=0.0035, respectively), compared to BCL2L12-positive patients, showing that the majority of the leukemia patients who achieved partial or complete response to chemotherapy were BCL2L12-negative. *Conclusions.* Although further investigations are needed to reveal the exact mechanism of *BCL2L12* action and to elucidate its role in AML, our results suggest that *BCL2L12* mRNA expression could be considered as a new independent marker for prognosis and chemotherapy response in AML.

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ANTAGONIZATION OF THE MATURATION BLOCK MEDIATED BY THE LEUKEMIA-SPECIFIC FUSION PROTEIN AML1/ETO

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Background. The leukemia-specific AML1/ETO fusion gene regulates the gene expression of myeloid cells through different mechanisms, particularly through epigenetic repression of AML1/RUNX1 target genes recruiting histons deacetylases (HDACs) and DNA metyltransferases (DNMTs). The factors contributing to the differentiation block in this leukemia thus probably include also dysregulated AML1/ETO target genes and cooperating oncogenes (e.g. c-kit). *Aims.* To study the contribution of the *in vivo* AML1/ETO target gene LAT2 (an adaptor molecule heavily repress in different AML/ETO positive cells) and the c-kit oncogene to the AML1/ETO-mediated block in differentiation in the t(8;21) positive AML. *Methods.* A Ponasterone-A (PonA) inducible system in the U937 cell line, 9/14/18-U937 was employed. AML1/ETO was induced by adding PonA 5µM to the cell culture. Cells were differentiated for 24 hrs with 10 nM PMA. Flow cytometry for CD11b was performed. Treatment of Kasumi-1 cells: decitabine (DAC) was added in three pulses every 24hrs (changing medium every day). Concentrations used were 50 nM, 100 nM and 200 nM. At 48hrs ATRA 1µM or Vitamin D3 250 ng/mL were added. Medium was changed every day; cell growth and viability were also assessed every day. Dasatinib was added in two pulses, at 0 and 48 hours (after changing medium), at 0.1 nM, 1.5 nM, 10 nM and 100 nM. Viability and cell growth were assessed at 24, 48 and 96 hours. A LAT2 shRNA 'knock-down' model in U937 (Tessarz *et al.* 2007) was treated for 48 hrs with 10nM PMA and multiple other differentiation inducers.

Table. Laboratory data.

	Age y / gender	Latent Period mo	Therapy for CLL	FAB Typ+ MDS/ AML	Cytogenetic	Therapy MDS/ AML	Survival Mo
1	71/M	24	FC(6 cycles)	M5	46,xy,t(3;7)(q26;q21)[23]	Chemo	1
2	54/M	10	CHOP, FMD, FC	M2	46,xy,del 5(q23;q33),-18,+mar(cp2)/46xy,del(5)(q23;q33),-11,-18,+3mar[cp2]/45xy,del(5)(q23;q33),-11,-18,-21,+3mar [14]/46,xy	Chemo	2
3	69/M	5	FC	RAEB2	46,xy,del(5)(q13;q33),t(11;17)(q21;q25),-20,+mar [13]/46,xy [2]	support	8
4	49/F	14	CHOP, FC	M4	43-46,XX,-5,-13,-18,+3mar [cp20]	chemo	4

Results. Conditional expression of AML1/ETO led to a partial decrease in PMA-induced differentiation of U937 cells. The treatment of Kasumi-1 with the DNMT inhibitor DAC resulted in a dose-dependent cell growth inhibition, reduction of viability and partial differentiation (decrease of CD11b expression). An enhancement of this effect

could not be seen with the combined treatment of 5-aza-2'-deoxycytidine (DAC) and 1,25-OH-Vitamin D3 or ATRA, known inducers of differentiation. The functional role of LAT2 in monocytic differentiation was investigated in a LAT2 shRNA "knock-down"model. From nine different differentiation inducers studied, only Phorbolester (PMA, 48 hrs, 10 nM) showed a modest but statistically significant differentiation effect on LAT2-deficient U937 cells when compared to control cells. Treatment of the Kasumi-1 cell line (c-kit-Mutation N822K) with dasatinib showed an inhibition of the autophosphorylation of c-kit, though no clear differentiation of the cells was achieved. *Summary/Conclusions.* Conditional expression of AML/ETO blocks PMA-induced differentiation of 9/14/18 U937 cells. The block in differentiation of the AML1/ETO-positive, LAT2-negative Kasumi-1 cell line could be antagonized through treatment with decitabine, though not with the c-kit inhibitor dasatinib. LAT2 (upregulated during monocytic differentiation) may have a functional role in blocking differentiation of AML cells. These results are being followed by studies in an AML1/ETO-positive, LAT2 negative blasts retrovirally overexpressing LAT2.

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PROGNOSTIC IMPACT OF ANGIOPOIETIN-2 (ANG-2) IN ACUTE MYELOID LEUKEMIA

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Introduction. Angiogenic factors play an essential role in normal and pathologic angiogenesis. The angiopoietins act as essential regulators in this process. The aim of this study is to detect the expression of angiopoietien -2 (Ang-2) in association with vascular endothelial growth factor A (VEGF-A) in patients with acute myeloid leukemia (AML) and its impact on prognosis and overall survival of patients. *Subjects and Methods.* Forty newly diagnosed AML patients as well as ten normal bone marrow samples were subjected to quantitative real time polymerase chain reaction (RQ-PCR) analysis for detecting the expression of Ang -2 as well as enzyme linked immunosorbant assay (ELISA) for detection of VEGF level. *Results*. This study revealed that high pretreatment levels of Ang-2 and VEGF-A indicate an unfavorable prognosis in AML. Expression of Ang-2 (hazarad ratio 16.8, 95% CI 3.4-82.6 P<0.01) and cytogenetic analysis (hazard ratio 4.5, 95% CI 1.3-15.3, P<0.01) were considered as independent prognostic factors for overall survival, the prognostic significance of Ang -2 expression was more obvious in patients with intermediate risk cytogenetics. In this study, Ang-2 expression was high in 21/40 of AML patients (52.5%) while 19/40 patients (47.5%) had low Ang-2 expression. In the control group, Ang-2 expression was low (<102 copies/mL) and VEGF level was <200 pg/mL. Ang-2 expression was not significantly correlated with any of the clinical or hematological features of AML patients (P>0.05). However, statistically significant association was observed between Ang-2 and CD34, VEGF-A level as well as cytogenetic risk groups. Regarding the clinical outcome of AML patients, out of the 19 patients with low Ang-2 expression group 73.7% showed good response to therapy and achieved CR while 26.3% showed poor response to therapy and failed to achieve CR (relapsed or died). On the other hand, among the 21 patients in the high Ang-2 expression group, 14.3% showed CR while 85.7% showed poor response to therapy and failed to achieve CR. Factors that unfavorably influenced the prognosis were the presence of leukemic burden, positive expression of CD34, high level of VEGF, high Ang-2 mRNA expression as well as cytogenetic risk group. *Conclusion.* high pretreatment levels of Ang-2 predict unfavorable disease outcome in AML. High expression of Ang-2 is associated with high VEGF, supporting the possibility of using angiogenesis inhibitors as an additional novel therapeutic strategy for this disease

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HIGH EXPRESSION OF AURKA AND B IS ASSOCIATED WITH UNFAVORABLE CYTOGENETIC ABNORMALITIES IN *DE NOVO* ACUTE MYELOID LEUKEMIA

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Acute myeloid leukemia (AML) is a malignant disease characterized by the excessive proliferation of myeloid precursors in bone marrow. Although chromosomal translocations have been associated with treatment outcome, the relationship between specific chromosomal translocations and pathways regulating mitosis have been not been studied. Aurora kinases play a critical role in regulating of mitosis and cell division, and their overexpression has been observed in many solid tumors, but little is known about their expression in AML. We have investigated AURKA and B expression in 70 cases of *de novo* AML by Real Time PCR. Using an arbitrary cut-off (≥7, for AURKA and <7, for AURK), we observed that 70% of cases with expression higher than the cut-off presented unfavorable cytogenetic abnormalities. No significant differences were found among age, sex and platelets count. However, we detected that the AURKA$^+$ group had 6-fold increase of the risk of presenting high WBC count than AURKA- (OR: 5.6; 95%CI 1.1 to 26.6, P=0.03). This result was not altered after adjustment for age, sex and FLT3-ITD mutations (OR: 7.1; 95%CI 1.22 to 41.2, P=0.02). Similar results were observed in AURKB+ group (OR unadjusted: 4.1; 95%CI 1.16 to 14.61, P=0.02; OR adjusted: 4.7; 95%CI 1.22 to 18.53, P=0.02). Taken together, we provide evidence that overexpression of AURKA and B may correlate with some factors known to be associated with specific translocations which previously have been shown to confer poor prognosis in *de novo* AML.

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CORRELATION OF ATP-BINDING CASSETTE (ABC) TRANSPORTERS PGP, BCRP AND ABCB6 EXPRESSION TO RESPONSE TO INDUCTION CHEMOTHERAPY IN ACUTE MYELOID LEUKEMIA PATIENTS

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Background. At least 20% of patients with acute myeloid leukemia (AML) exhibit primary resistance to induction chemotherapy. Classical multidrug resistance (MDR) is caused by upregulation of membrane-resident transporters that efflux chemotherapeutic drugs from tumor cells, like the ABC transporter P-glycoprotein (P-gp, ABCB1), which has been associated with lower remission rates and shorter survival. Other ABC proteins, such as BCRP (ABCG2) may have clinical significance in AML. A recently discovered ABC transporter ABCB6 has also been implicated in MDR development. ABCB6 was overexpressed in a panel of 60 cancer cell lines and has been correlated with increased resistance to chemotherapeutic drugs. No data are so far available about ABCB6 involvement in MDR development in AML. *Aim.* To evaluate the level of Pgp, BCRP and ABCB6 gene expression in bone marrow (BM) samples from AML patients at diagnosis, in relation to response to induction chemotherapy. *Methods.* We studied 34 AML patients admitted to our Department; BM samples at diagnosis were obtained for routine analysis. Non-involved BM samples obtained from Non- or Hodgkin Lymphoma patients for staging purposes were used as control samples. Total RNA was extracted and was reverse transcribed to cDNA. Pgp, BCRP and ABCB6 expression was assessed by quantitative Real Time RT-PCR using SYGR and the Lightcycler Instrument. Gene transcripts were normalized to β-actin and differences in expression were calculated using the ΔΔCT method. Mann Whitney's test was used for statistical analysis. *Results.* Among the 34 patients, 20 were males with a median age of 58 years (range 28-77). Twenty eight patients were *de novo* AML and 6 developed AML after preceding myelodysplastic syndrome (MDS); FLT3-ITD mutations were present in 25% of checked patients. Median value for bone marrow blasts at diagnosis was 78% (range 21-98). Most patients received idarubicin and infusional cytarabine as induction treatment; 22 of them achieved remission, 8 were primary resistant and 4 were not evaluable mostly due to early toxic death. Analysis of Pgp, BCRG and ABCB6 data showed a trend for increased expression in primary resistant patients (median values 8,38, 1,07 and 0,59 respectively) compared to those who achieved remission (median values 1,5, 0,24 and 0,33 respectively), however the differences reached borderline significance, most likely due to the small sample number. We also found a trend for Pgp overexpression in patients who developed AML on a preexisting MDS compared to *de novo* AML patients (median value: 7 and 1,51 respectively), but this difference was of borderline statistical significance as well. The expression levels of the three transporters did not correlate with the percentage of BM blasts at diagnosis. *Conclusions.* The present study is in concordance with the previously reported prognostic significance of Pgp expression on achievement of

complete remission in AML patients. The recently discovered ABCB6 transporter could also be implicated in development of MDR in AML, however we plan to analyse more primary refractory patients in an effort to confirm our hypothesis.

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TYROSINE KINASES EXPRESSION PROFILES IN PATIENTS WITH DIFFERENT GENETIC SUBTYPES OF ACUTE MYELOID LEUKEMIA

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Background. Tyrosine kinase plays an important role in regulation of cell growth, proliferation and survival. *FLT3, KIT, JAK2, JAK3* and *PDGF* have been proved to contribute to leukemogenesis in acute myeloid leukemia (AML). However, the expression levels in most tyrosine kinases are limited in AML. Clarify the gene expression of tyrosine kinases among different genotype of AML may help to know the molecular mechanism of leukemogenesis, and potentially improve the target therapy in the future. *Aims.* Clarify the expression profiles of receptor tyrosine kinases in leukemic blasts in different subtypes of AML. *Patients and Methods.* Patients. Bone marrow specimens were collected from 48 AML patients after inform consent. This study was approved by the Institutional Review Board of National Taiwan University Hospital. Cell line. Cell lines (K562, HL60, U937, Kasumi-1 and THP-1) were cultured with RPMI1640 and 10% fetal calf serum. RNA extraction and RT-PCR RNA extracted from AML patients bone marrow samples with QIAamp RNA Blood Mini Kit, and was treated with DNase and quantitation with NanoDrop® ND-1000 Spectrophotometer, then RNA was converted to cDNA with reverse transcriptase Omniscript RT Kit. Taqman assay was designed based on the genomic sequence. Samples were run in triplicates on a 7500 real-time PCR system (ABI). Nine tyrosine kinases (*DDR1, FLT3, TYRO3, ROR2, INSRR, MATK, MST1R, HCK* and *LYN*) were checked with Taqman RT-PCR technique. Gene expression was normalized to glyceraldehyde phosphate dehydrogenase (*GADPH*) expression and calculated relative to normalized expression in control samples.

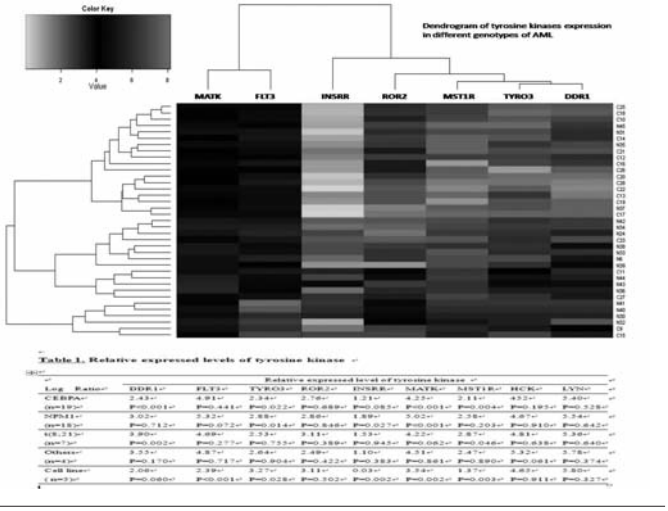

Table 1. Relative expressed levels of tyrosine kinase

	Relative expressed level of tyrosine kinase							
Leg Ratio	DDR1	FLT3	TYRO3	ROR2	INSRR	MATK	MST1R	HCK	LYN
CEBPA (n=19)	2.43 P=0.001	4.91 P=0.441	2.34 P=0.022	2.76 P=0.889	1.23 P=0.085	4.23 P=0.001	2.11 P=0.004	4.32 P=0.195	3.40 P=0.328
NPM1 (n=18)	3.02 P=0.712	2.32 P=0.072	2.88 P=0.014	2.86 P=0.848	1.89 P=0.027	3.02 P=0.001	2.38 P=0.203	4.87 P=0.910	3.34 P=0.842
t(8;21) (n=7)	3.80 P=0.002	4.89 P=.277	2.33 P=0.753	3.11 P=0.389	1.33 P=0.943	4.22 P=0.082	2.87 P=0.046	4.81 P=0.938	3.36 P=0.840
Others (n=11)	3.33 P=0.170	4.87 P=0.717	2.64 P=0.904	2.49 P=0.422	1.10 P=0.383	4.31 P=0.861	2.47 P=0.590	3.32 P=0.081	3.78 P=0.374
Cell line (n=3)	2.06 P=0.080	2.39 P=0.001	3.27 P=0.028	3.11 P=0.703	0.93 P=0.002	3.34 P=0.002	1.37 P=0.003	4.82 P=0.911	3.80 P=0.327

Figure 1. tyrosine kinases expression profiles in AML.

Results. Tyrosine kinase expression in cell lines. Five cell lines (K562, HL60, U937, Kasumi-1 and THP-1) were analyzed for the tyrosine kinase expression. Relatively low expression of FLT3, TYRO3, INSRR, MATK and MST1R was found in cell line samples. Even there were the same cytogenetic changes such as Kasumi-1(AML-ETO) compared with t(8;21) human bone marrow samples, the result also showed significant difference between cell line and bone marrow samples. Tyrosine kinase expression in AML bone marrow samples. There are 48 AML patient (25 men/23 women) enrolled in this study(CEBPA 19, NPM1 18, t(8;21) 7, Other 4). The results were shown as Table 1. The relative expressed levels of tyrosine kinase in cell lines are significantly different from the mononuclear cells in AML patients' bone marrow. The *DDR1* kinase and MST1R kinase are expressed relatively high in the patients with t(8;21), but low in patients with *CEBPA*. *TYRO3* kinase expressed high in NPM1, but low in *CEBPA*. *INSRR* kinase expressed high in *NPM1*. *MATK* kinase expressed high in *CEBPA* and *NPM1*. Expression of *HCK* and *LYN* is no difference among different subtype of leukemia. The expression profile of tyrosine kinases were analysed with cluster analysis, the dendrogram of tyrosine kinases was shown as Figure 1. *Summary/Conclusions.* AML is known with many genetic subtypes which are highly correlated with treatment response and disease survival. Molecular mechanism of AML remains to be investigated. Tyrosine kinase expression profiles in different genetic subtypes potentially help us to explore the complex signal transduction network in the cancer cells.

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CONSEQUENCES OF ALTERED DNTP LEVELS FOR GENOME STABILITY IN HEMATOPOIETIC CELLS

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Cells have evolved numerous mechanisms to ensure proper maintenance of their genetic information. These mechanisms are particularly important during cell division, since self-enforcing accumulation of mistakes in the genome is thought to contribute to the development of somatic genetic disorders, particularly cancers. The ribonucleotide reductase (RNR) is a tetramer composed of two non-identical homodimers; R1:R2 or R1:R2b. It catalyzes the *de novo* synthesis of deoxyribonucleotides (dNTPs) to provide a strictly balanced supply of dNTPs required for DNA synthesis and repair. The activity of this enzyme is under exquisite transcriptional and post-transcriptional control to avoid unbalanced dNTP pools that could lead to misincorporation of nucleotides into DNA, mutations, and cell death. Several observations suggest that changes in RNR activity might be an important factor in tumorigenesis (Angus *et al.,* 2002; Fan *et al.,* 1998). In fission yeast it has been demonstrated that the inability to degrade the RNR inhibitor Spd1 results in reduced dNTP pools and elevated mutation rates (Holmberg *et al.,* 2005). Furthermore, fission yeast cells carrying a mutation that relaxes the allosteric feedback inhibition site of RNR exhibit elevated dNTP pools and do also display an increased mutation rate (Olaf Nielsen, unpublished data). In order to investigate whether deregulated levels of nucleotide pools play a role in cancer development, it will be tested to what extend modulation of RNR activity collaborates with various defined genetic lesions in three mouse models of human leukemia. These model systems rests on the ability of retrovirally transduced hematopoietic cells to repopulate the entire hematopoietic system of a lethally irradiated recipient mouse. To test whether modulation of dNTP pools affects tumor latency in any of the two mouse cancer models *Cebpalphap30/p30* or *MLL-AF9,* HSCs/hematopoietic progenitor cells (HPCs) will be transduced with retroviruses aimed at deregulating RNR activity. The deregulation of RNR will be obtained by overexpression of the three different subunits of RNR. To avoid the tight allosteric feedback inhibition of RNR that during overexpression potentially could hamper the increased acitivity of the enzyme a point mutation in the feedback inhibition site of R1 will be introduced. Furthermore, down-regulation of RNR activity in tumor-prone HSCs/HPCs will be achieved in two ways. The activity of R1, R2, and R2b will be down-regulated through expression of shRNAs, but also by expression of the RNR inhibitory yeast proteins Spd1, Sml1, and Dif1. Since RNR subunits are highly conserved, we expect these inhibitors to work across species barriers according to preliminary results. Results thus far shows that mice transplanted with *MLL-AF9* HSCs/HPCs with R1 knockdown lead to a prolonged latency compared to control mice. Since RNR activity may be limiting for progression to and through S-phase, R1 knockdown in *MLL-AF9* HSCs/HPCs might lead to inhibition of proliferation or to a more modest level of dNTP that is not compatible with oncogenic transformation. Both options are in agreement with the increased latency period in MLL-AF9 R1 knockdown transplanted mice.

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THE LOW EXPRESSION OF EPH A4 AND B4 HAS NO PROGNOSTIC IMPACT IN AML PATIENTS

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Background. Eph receptors represent the large subfamily of receptor tyrosine kinases (RTKs), named for its expression in an erythropoietin - pro-

ducing human hepatocellular carcinoma cell line. Both Eph receptors and their ligands-ephrins can be divided into two subclasses, A and B, on the basis of sequence homology, structure, and binding affinity. They play key roles in diverse biological processes: the development of the nervous and vascular system. The up- regulation of Eph receptors has been reported in various solid tumors, including breast, lung, colorectal, and esophageal cancers. This over- expression correlates with tumor invasiveness, vascularization, and metastatic potential. There is very limited data concerning Eph expression in haematological malignancies. *Aim.* This study aimed to investigate the expression of Eph A4, Eph B2, and Eph B4 in non- M3 acute myeloid leukemia (AML) patients, compare the expression levels with normal controls, and determine their prognostic significance. *Methods.* Bone marrow samples from 83 newly diagnosed non- M3 AML patients and 8 controls for comparison were quantified by real time RT- PCR, and the comparative cycle threshold (Ct) method was used to determine the relative expression for Eph A4, Eph B2 , Eph B4 to ABL control gene. All AML patients were treated according to Polish Acute Leukemia Group protocol. *Results.* The total cohort of 83 cases was composed of 49 males and 34 females with a median age of 47.9 years (range: 19-84 years). The relative expression levels of Eph A4 were significantly lower in AML patients as compared to healthy controls (ΔCt 2.871±1.54 *vs.* ΔCt -0.647±0.781, t=6.341, P<0.001 respectively). Comparison between leukemic and normal samples showed that Eph B4 expression was also significantly lower (ΔCt 1.890±1.575 *vs.* ΔCt 0.586±1.023, t=2.289, P=0.024 respectively). Lower expression was also found for Eph B2, but did not reach statistic significance. There was no statistical correlation between the expression of Eph A4, Eph B2, and Eph B4, and patients age, sex, AML subtype according FAB classification, risk group, complete remission rate, and overall survival. *Conclusions.* Our preliminary results showed that in AML patients expression of Eph A4 and Eph B4 was significantly lower than in healthy controls. However in our study low expression of Eph A4 and Eph B4 had no prognostic impact on clinical course of AML.

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SOLUTE CARRIER FAMILY 17 MEMBER 1(SLC17A1) IS A NOVEL GENE CONTROLLING ARA-C CYTOTOXICITY

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Cytosine arabinoside (Ara-C), a structural analogue of deoxycytidine, is a major drug in the treatment of acute myeloid leukemia (AML). Therefore, emergence of resistance to Ara-C is a major problem in AML treatment. To investigate novel genes associated with resistance to Ara-C, whole genome SNP chip analysis was performed. Even though no genes were found that are significantly associated with Ara-C metabolism, 12 regions of loss of heterozygosity (LOH) were found in induction failure group. SLC17 family gene and HIST1H family gene were located in the majority of LOH found in induction failure group. Since SLC29A1, a nucleoside transporter, belonging to solute carrier family genes directly affects Ara-C mediated apoptosis in recent reports, we chose SLC17A1 that has co-transport activity and performed functional analysis of SLC17A1. Its expression levels were examined in 4 AML cell lines (HEL, NB4, KG-1 and ML-1) using RT-PCR. Despite a difference in its expression levels among 4 cell lines, it seemed to be constitutively expressed. To detect changing patterns of SLC17A1 in AML cells surviving Ara-C treatment, 4 cell lines (HEL, NB4, KG-1 and ML-1) underwent on and off treatment of 10^{-8} M of Ara-C for 2 and 4 cycles. Expression of SLC17A1 was decreased more than SLC29A1 expression in the same condition. To examine whether the expression levels of SLC17A1 was associated with Ara-C resistance, Ara-C resistant HEL cell was established *in vitro* and *in vivo* (HEL-R1 and HEL-R1-vivo). SLC17A1 mRNA expression of both resistant cells is decreased or less increased than parental HEL cells with Ara-C treatment. Reduction of SLC17A1 mRNA by shRNA of SLC17A1 effectively suppressed Ara-C mediated apoptosis. Our results suggested that the function of SLC17A1 gene was similar to that of SLC29A1. Taken together, the expression levels of SLC17A1 affected the cytotoxic effects of Ara-C. Thus, our results suggest SLC17A1 as a novel gene controlling Ara-C mediated apoptosis.

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GENE EXPRESSION PROFILES IN RESVERATROL APPLIED ACUTE PROMYELOCYTIC LEUKEMIA CELLS

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Background. Resveratrol, (3,5,4'-trans-trihydroxystilbene) is a natural product found in plant constitutes such as grape skin. It has shown significant cytotoxic and apoptotic effects on various types of cancer cells with no harm to normal healthy cells. Resveratol inhibits tumor initiation, promotion, and progression. However the mechanisms of resveratrol induced cell death is not well-known. We have previously showed cytotoxic and apoptotic effects of resveratrol on acute premyelocytic leukemia (APL) cells. APL is a hematological malignancy characterized by increased number of clonal population of hematopoietic progenitor cells. *Aims.* In this study, we aimed to show molecular mechanisms of resveratrol induced apoptosis by examining the changes in expression profiles of human cancer signalling pathway genes in HL60 APL cells exposed to resveratrol. *Methods.* Effective concentrations of resveratrol on HL60 cells were determined by XTT cell proliferation assay. Total RNAs were isolated from HL60 cells exposed to 10 and 50 μM resveratrol, converted to cDNA, and changes in expression levels of 84 genes involved in apoptosis, metastasis, angiogenesis, invasion, adhesion, tumor supressors, and transcription factors by PCR array. *Results.* Resveratrol has shown antiproliferative effect on HL60 cells in a dose dependent manner. There were 15 and 45% decreases in cell proliferation in response to 10 and 50 μM resveratrol in HL60 cells as compared to untreated controls, PCR array results demonstrated that there were more than 3-fold increase in expression levels of 24 and 36 genes in HL60 cells treated with the same concentrations of resveratrol as compared to control, respectively. On the other hand, there were 6 genes whose expression levels were decreased more than 4-fold in response to 10 and 50 μM resveratrol, respectively. We observed significant increases in expression levels of p53 in a dose-dependent manner which is not detected in control group. The most significant increases were observed in apoptotic genes (e.g. Bax, Tert), and decreases were observed in antiapoptotic genes (e.g. Bcl-2). Although, there were increases in expression levels of certain growth factor, antiapoptotic and metastatic genes, our whole data demonstrated that these concentrations of resveratrol inhibited cell growth and induced apoptosis in HL60 cells. *Summary/Conclusions.* In this study the mechanisms of resveratrol-induced apoptosis were demonstrated in detail. This *in vitro* data by being supported with clinical data may open the way of the potential use of resveratrol for acute premyelocytic leukemia patients.

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VASCULAR ENDOTHELIAL GROWTH FACTOR-A ACTED AS AN AUTOCRINE GROWTH FACTOR ON SOME OF ACUTE PROMYELOCYTIC LEUKEMIA CASES: RELATIONSHIP TO HYPER-LEUKOCYTOSIS

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Background. In acute promyelocytic leukemia (M3), a prominent production of vascular endothelial growth factor (VEGF)-A is reported, and an obvious angiogenesis is observed in bone marrow specimens; however, VEGF receptors (VEGFRs) are not expressed in general. We recently reported that an autocrine system of VEGF-A-system was observed in one M3 case (submitted in Leukemia and Lymphoma). *Aims.* In this report we analyzed the expression of VEGF and VEGFRs in our experienced M3 cases to clarify that in what kind of M3 VEGF-A autocrine system was demonstrated. *Patients, materials and Methods.* Patients, administered to our hospital from Nov. 1998 to Nov. 2009 and diagnosed with M3, were eligible. Bone marrow cells were obtained from informed patients, whose mononuclear cells were prepared with density-gradient sedimentation method. Cells were cultured short term for the elimination of an adherent cell-fraction. RNA was extracted from the prepared non-adherent mononuclear cells, and the expression of VEGF-A and VEGFRs was analyzed with reverse transcription-polymerase chain reaction (RT-PCR). When cells expressed VEGFRs with RT-PCR, they were analyzed on the protein level with fluorescent activated cell sorter. VEGF-A levels in

patients' sera and in the conditioned media from the cultured M3 cells were assayed with ELISA kit. When M3 blasts expressed VEGF-A and VEGFRs, a growth-inhibition effect by the administration of anti-VEGF-A-neutralizing antibody was assayed. *Results.* In all 21 cases examined, VEGF-A production was observed. VEGFR-1 and -2 were expressed in 3 cases, in all of which WBC count was above 20,000/μL at the onset of the disease (20,800, 21,500, and 71,400). Statistical significance was demonstrated on WBC count between VEGFRs positive group and negative one. When VEGF-A-neutralizing antibody was added to the blast cell-culture of these three patients, the cell-growth was significantly inhibited. *Conclusion.* These observations indicate that VEGF-A-autocrine system works on proliferation in a few of M3 cases with hyper-leukocytosis.

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ANALYSIS OF FLT3 MRNA EXPRESSION LEVEL IN ADULT AML

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Background. Although FLT3 mutations are analyzed in large number of AML patients, and their biological and clinical significance is well known, only a few studies are available regarding FLT3 mRNA expression. *Aims.* The aim of this study was to evaluate expression level of FLT3 gene in 44 adult AML patients (41 *de novo*, and 4 relapsed patients), and in 6 healthy donors. We examined the possible correlations between the FLT3 expression and other clinical characteristic, as well as with FLT3 mutational status. Also in 4 patients we examined the level of FLT3 during the course of the disease. *Methods.* Total mRNA was isolated from PBMCs and cDNA was prepared by reverse transcription. FLT3 expression level was quantified by real-time PCR method, using SYBR Green chemistry. Abl served as a housekeeping gene. Relative quantification analysis was performed using comparative Ct method (2-ΔΔCt), where ΔΔCt = ΔCt(sample) -dCt(healthy(median)). Relative quantity of FLT3 is expressed in relative units (RU). *Results.* FLT3 expression was significantly higher (mean 18,5±2,7, range 1,1-70,0) in AML patients, compared to healthy donors (mean 1,11±0,4, range 0,1-2,6)(P<0.001, U-test). Among patients with different FAB types and different karyotypes, FLT3 expression levels were very heterogeneous, showing no significant difference between groups. We also found no correlation between the level of FLT3 expression and other clinical parameters as age, white blood cell count, percentage of BM blasts and presence of CD34 positive cells. Regarding FLT3 mutational status, we found 4 FLT3/ITD patients and 6 FLT/D835 patients. Neither FLT3/ITD nor FLT3/D835 positive patients showed tendency towards higher level of FLT3 expression. Based on their karyotype, patients were classified into 3 prognostic groups; favorable (t(15/17) (n=18), inv16 (n=3), t(8;21) (n=1)), intermediate (normal karyotype (n=10)), and bad (n=10) prognosis. All 4 relapsed patients were t(15;17) positive, and were observed as a separate prognostic group. After implementation of the cut-off value for FLT3 expression, all of the patients with FLT3 expression above 20,0 RU, were considered to have high FLT3 expression. Significant association between prognosis and high FLT3 expression was found in both favorable and adverse prognostic group (P<0,001), but not among patients with normal karyotype. However, no influence of FLT3 expression on overall survival (OS) was found ((P=0,49; Log Rank), even when t(15;17) positive patients were excluded. Analysis of FLT3 expression levels during the course of the disease in 4 patients, showed rapid decline during CR to the level found in healthy controls. It remained low during continuous CR (2 patients), but increased during the time of relapse (2 patients), back to the level found at the onset of disease. *Conclusion.* Our study shows vide range of FLT3 expression levels and its heterogeneity regarding FAB, karyotype and other clinical parameters. There is also a significant association between high FLT3 expression level and prognosis in favorable and adverse prognostic groups. Although we didn't find association between FLT3 expression level and OS, we found strong association between high/low expression and relapse/complete CR. We suggest that, examination of FLT3 expression level in AML has been wrongly neglected both as prognostic marker and for MRD monitoring.

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METALLOPORYPHYRIN CONTROLS THE DIFFERENTIATION OF HUMAN PROMYELOCYTIC LEUKEMIC CELLS

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Backgroud. Heme oxygenase -1 (HO-1) is an inducible cytoprotective molecule, which displays antioxidant, anti-apoptotic and anti-inflammatory effects. HO-1 is well induced in leukemia. However, there is no report about role of HO-1 in leukemia.*Aims.* We investigated the involvement of HO-1 in the differentiation of HL-60 cells.*Methods.* HO-1 expression was measured by western blot and realtime PCR. Differentiation of HL-60 cells was determined by CD11b expression through flow cytometric analysis.*Results.* Dimethylsulfoxide (DMSO), a representative differentiation inducer of HL-60 cells, induced completely decrease HO-1 expression in a time-dependent manner, but increase CD11b, indicating that HO-1 might have negative function in DMSO-induced differentiation of HL-60 cells. Zinc protoporphyrin (ZnPP), a strong inhibitor of HO-1, induced differentiation of HL-60 cells, as evidenced by a marked increase in the expression of CD11b following ZnPP treatment. In contrast, treatment with cobalt protoporphyrin (CoPP), an activator of HO-1, decreased CD11b expression. ZnPP induced down-regulation of HO-1 protein expression in HL-60 cells, while CoPP induced up-regulation. In addition, ZnPP treatment caused a decrease in pRb and cyclin D1 expression and an increase in p21 and p27 expression. *Conclusions.* These results suggest that down-regulation of HO-1 might be necessary for DMSO-induced HL-60 differentiation. This study provides the first evidence that HO-1 plays an important role in DMSO-induced differentiation of HL-60 cells.

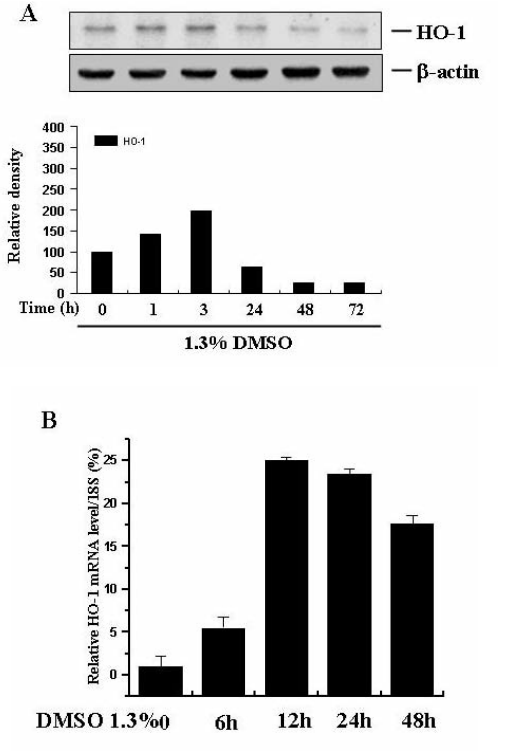

Figure. HO-1 down-regulation by DMSO

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EXPRESSION OF MULTIDRUG RESISTANCE GENES MDR1, MRP1, LRP AND BCRP IN CORRELATION TO THE PRESENCE OF FLT3-ITD MUTATION IN ACUTE MYELOID LEUKEMIA- PRELIMINARY RESULTS

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Background. Resistance to chemotherapy in acute myeloid leukemia (AML) is the most important clinical problem and is associated with the expression of the multidrug resistance (MDR) proteins: P-glycoprotein,

multidrug resistant related protein, lung resistance-related protein and breast cancer resistance protein encoded, respectively, by the MDR1, MRP1, LRP and BCRP genes. Moreover some gene mutations e.g. internal tandem duplication (ITD) of FLT3 receptor detected in about 25-35 percent of AML patients portend a poor prognosis. *Aims.* In this retrospective study we estimated expression of the MDR genes and the presence of FLT3-ITD mutation in patients with newly diagnosed, untreated AML. We divided all patients into two groups accordingly to the presence of FLT3-ITD mutation and we compared expression of MDR genes in these groups. We also investigated the relevance of tested genes and FLT3-ITD mutation with other known prognostic factors in AML: age, cytogenetic/molecular aberrations, *de novo*/secondary AML, white blood cell count (WBC), extramedullary involvement and type of AML according to the FAB classification. *Methods.* A total of 46 adult patients (21 women and 25 men) at median age 54 years (range 21-87) with newly diagnosed, untreated AML and 40 healthy donors were included in this study. Diagnosis of AML was based on standard morphological and immunophenotypical criteria according to the WHO classification. There were sixteen FLT3-ITD positive patients (34.7%) and thirty FLT3-ITD negative patients (65.3%). DNA was extracted from bone marrow or peripheral blood samples and used to detect FLT3-ITD mutation with PCR method. RNA was extracted from the same samples and RQ-PCR method was performed for assessment of expression of MDR1, MRP, LRP and BCRP genes. Parameters of RQ-PCR reaction were established based on the Europe Against Cancer protocol. Every tested sample was amplified simultaneously for the presence of control ABL gene for standardization of mRNA level for tested genes. Results of the expression of tested genes were presented as coefficients calculated with an intermediate method according to Pfaffl's rule. *Results.* Significant higher expression of MDR1 gene was found in patients without FLT3-ITD mutation in contrast to the patients with FLT3-ITD mutation (0,1949 *vs.* 0,0701, P=0.002). For MRP, LRP, BCRP genes we did not find significant differences in expression between two groups though we observed much higher expression of BCRP gene in patients without FLT3-ITD. When adjusted for other prognostic factors, only WBC was significantly higher in FLT3-ITD positive group of patients: 3,7-227×10^9/L, median 74 *vs.* 0,5-118×10^9/L, median 11 in FLT3-ITD negative group of patients (P=0,009). We did not observe significant differences in complete remissions and relapses rates and disease free survival and overall survival between groups. *Conclusions.* Significantly higher expression of MDR1 gene was observed in FLT3-ITD negative group of patients. It seems that the presence of FLT3-ITD mutation and high expression of MDR1 gene are independent high risk factors which negatively influence results of AML therapy.

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MLL-AF6, A POOR PROGNOSIS FACTOR OF ADULT AML. ROLE OF MRD MONITORING USING QUANTITATIVE REAL TIME PCR

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Background. AML is an heterogeneous disease. Their association to a specific molecular rearrangement allows for a better prognostic stratification. The mixed lineage leukemia (MLL) gene is frequently translocated. MLL is a protooncogene with a transcriptional role that relies on its ability to covalently modify chromatin through its histone H3-K4 methyltransferase activity. Its oncogenic role is associated with deregulated expression of .the master key regulator HOX genes. Indeed, MLL can be fused to more than 50 different partners. In adult AML, AF9 (MLLT3) is the most frequent partner. AF6 (MLLT4) is a rarer partner whose prognosis and role of monitoring are less known. *Patients and Methods.* During the last 10 years, we have monitored in the same laboratory, 9 AML patients with a RT-PCR detectable MLL-AF6 translocation at diagnosis. They were 5 males and 4 females, median age 34 (range 16-56). They were treated into or according to multicenter treatment trails ALFA 9802 (5 pts), ALFA 0702 (1 pt), LAM-EORTC (1 pt) or CHA (3+7) (2 pts). Quantification of MLL-AF6 transcript was performed according to the ELN recommendations for real time quantitative PCR. *Results.* The first patient died of an intracardiac granulocytic sarcoma at D7 post induction therapy. Remission has been obtained for all other patients. A high dose AraC consolidation was performed and well tolerated in all patients except one with severe cerebellitis sequels. The 7 remaining patients underwent an early allogenic HSCT (between M3 and M5) for 5 patients with a geno or pheno identical donor, and a later one (M8 and M15) for the 2 remaining patients for which a donor was difficult to identify. Six out of 8 patients relapsed at M4, M6, M8, M8, M9 and M16 and did not experience any durable second remission although 2 of them underwent a second allogenic HSCT. At the present time, 6 out of the 9 patients are deceased (J7, M14, M16, M19, M25, M47). One is alive and in relapse at M14 post HSCT. Two patients are still in remission, one with a severe HDAC neurologic toxicity. One patient is alive and well at M18. MLL-AF6 transcript quantification was performed according to the ELN recommendation using primers and probes which have already been published (C. Scholl, Haematologica 2005, 90, 1626). Sensitivity was 10^{-5}. For patient monitoring, we have tested 128 serial MRD patient samples. In all cases, a complete molecular response was observed on blood samples. The molecular relapse anticipated the haematological relapse by 2 to 4 months in all cases. None of the patients, who experienced a relapse and a haematological second remission, had a second molecular remission. *Conclusion.* The prognosis of MLL-AF6 adult AML sub-group, in this small series, is poor. Minimal residual disease monitoring of MLL-AF6 transcript allows foreseeing the future of these patients. These observations should incite: 1) to treat patients before haematological relapse, at the stage of a confirmed molecular relapse, 2) to look for targeted therapeutic strategies based on MLL oncogenicity newly acquired knowledge to render MRD monitoring really useful.

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CCAAT/ENHANCER BINDING PROTEIN ALPHA (C/EBPA) GENE EXPRESSION LEVELS AS A PROGNOSTIC MARKER IN 49 EGYPTIAN AML PATIENTS

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Background. The transcription factor CCAAT/enhancer binding protein Alpha (C/EBPA) is a myeloid specific transcription factor that regulates the balance between cell proliferation and differentiation in haematopoietic and non-hematopoietic tissues. C/EBPA plays a major role during the commitment of hematopoietic stem cells towards granulocytic and monocytic differentiation. Impairments in C/EBPA signalling such as reduced mRNA and protein expression are often observed in human myeloid leukemias which may subsequently contribute to leukaemic transformation (Trivedi *et al.*, 2007). In the last few years, various mechanisms have been suggested through which C/EBPA is negatively regulated in certain AML FAB subtypes *Aim.* Studying the heterogeneity of C/EBPA m-RNA expression among different FAB subtypes of newly diagnosed AML patients, as a first step in unraveling the impact of newly discovered genetic abnormalities on the pathophysiology of AML. *Methods.* Fourty nine AML patients were enrolled in the study, 19(38.8%) males, 30(61.2%) females and mean age 32 years (range 2-77). Controls included 20 healthy subjects, 11(55%) males and 9(45%) females with mean age 33 years (range 16-59). The diagnosis of AML was established according to morphology and immunophenotyping (FAB classification). The quantitative assessment of C/EBPA gene expression in AML patients at diagnosis and healthy subjects was performed using RT-PCR *Results.* CEBPA expression levels among the Healthy population and AML patients had an average expression of (1.41±1.14 & 0.52±1.13 respectively, P=0.001). The majority of our study population (40/49) had Low CEBPA expression levels (range 0.001-0.547). Few (6/49) cases showed Intermediate expression levels (range 0.812-1.866) while only 3 cases showed High expression level (range 2.828-5.278). Clinically, Low expressing subgroup had lower DFS and ORS rates than Intermediate and High subgroups with very high statistical significance (P=0.001* and P=<0.001* respectively) *Conclusion.* Using real-time PCR we were capable of defining three prognostic subgroups, that is, high, intermediate and low C/EBPA mRNA expressing patients. Accordingly, C/EBPA expression analysis should be carried out on a larger cohort prior to treatment to predict AML patient's outcome

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IMPACT OF CYTOGENETIC ANALYSIS AND MOLECULAR SCREENING ON RISK STRATIFICATION AND FOLLOW- UP IN AML CHILDREN IN SLOVAKIA

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Background. AML is aggressive, phenotypically and genetically heterogenous clonal disease of hematopoietic progenitor cells with a great

molecular variability. New WHO classification 2008 divides *de novo* AML according to cytogenetic and molecular prognostic and predictive markers. Recently, it is increasingly possible to identify a subgroup of poorer risk patients among those with normal karyotype AML. *Aims.* The aim of our study was to identify prognostically important molecular markers in children with AML, to stratify patients with normal karyotype and to monitor the disease according the genetic findings. *Methods.* In years 2006-2009 we analysed samples of bone marrow and peripheral blood of 20 children with *de novo* AML by conventional cytogenetic analysis, fluorescence *in situ* hybridisation and arrayCGH. The molecular analysis was performed on the cDNA level, with the restriction analysis of PCR products (FLT3-TKD), conventional PCR (MLL-PTD, NPM1mut, FLT3-ITD) and quantification RT-PCR method (expression of fusion transcripts, BAALC, WT1). *Results.* Samples from 20 children with AML were analysed. With the usage of conventional cytogenetics, FISH and arrayCGH, abnormal karyotype was revealed in 14 patients (70%): t(8;21) in 3/14, t(15;17) in 2/14, t(6;11) in 1/14, t(11;19) in 1/14, inv(16) in 1/14, t(X;11) in 1/14, t(6;9) in 1/14, +8 in 1/14, del(7q) in 1/14, complex karyotype in 2/14 patients. Further analysis revealed FLT3-ITD in 5/20 (25%), FLT3-TKD in 3/20 (15%), NPM1mut in 2/20 (10%) and MLL-PTD in 1/20 (5%), overexpression of WT1 gene in 15/20 (75%) and overexpression of BAALC in 13/20 (65%) patients. *Conclusions.* Wide cytogenetic and molecular screening helped us to find at least one genetic marker in all 20 patients for later follow-up and risk stratification. 6/20 (30%) patients died of the disease progression. These patients revealed following genetic aberrations: a, t(X;11); b, t(6;11); c, inv(16); d, FLT3-ITD and MLL-PTD; e, del(7q) and FLT3-TKD; f, +8 and FLT3-ITD.

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COEXISTENCE OF ALTERNATIVE MLL-SEPT9 FUSION TRANSCRIPTS IN AN ACUTE MYELOID LEUKEMIA WITH T(11;17)(Q23;Q25)

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Abnormalities of 11q23, resulting in fusion of the mixed lineage leukemia (*MLL*) gene with numerous translocation partners, are found in primary acute lymphoblastic leukemia and acute myeloid leukemia, as well as in secondary, topoisomerase II inhibitor-related leukemia To date, more than 50 *MLL* fusion partners have been identified. Five of these, *SEPT2, SEPT5, SEPT6, SEPT9*, and *SEPT11*, code for septins, which are an evolutionarily conserved family of genes with 14 members identified so far. Septins are conserved GTP-binding proteins that assemble into homo- and hetero-oligomers and filaments involved in several processes of cell division and cellular integrity, including polarity establishment, maintenance of membranar dynamics, vesicle trafficking, exocytose, cytoskeleton remodeling, and apoptosis. *SEPT9* (MSF or AF17q25) has previously been cloned as a fusion partner of *MLL* in *AML* with t(11;17)(q23;q25). To our knowledge, *MLL-SEPT9* fusion transcripts have so far been characterized at the molecular level in only nine myeloid neoplasia patients. The *MLL-SEPT9* rearrangements previously reported involve fusions between MLL exon 8 and *SEPT9* exon 3 (type I fusion transcript) (five cases), MLL exon 8 and *SEPT9* exon 2 (type II fusion transcript) (two cases), and MLL exon 7 and *SEPT9* exon 2 (type III fusion transcript) (two cases), with each case presenting only one fusion transcript variant. In this work, we aim to characterize at the RNA level an acute myeloid leukemia patient with a t(11;17)(q23;q25) and a MLL rearrangement demonstrated by FISH. Molecular analysis by RT-PCR led to the identification of two coexistent in-frame *MLL-SEPT9* fusion transcripts (type I and type II), both of them previously described isolated in the literature. The shorter (type I) and longer (type II) transcripts differ by the absence of SEPT9 exon 2 in the former, presumably by alternative splicing. Real-time quantitative RT-PCR analysis of the alternative transcripts showed that the relative expression of the *MLL-SEPT9* type II was 1.88 fold higher than the relative expression of the *MLL-SEPT9* type I. This is the first description of a *MLL-SEPT9* fusion resulting in coexistence of two alternative splicing variants, each of which previously found isolated in myeloid leukemias. Further studies are warranted to evaluate the functional and biological significance f these two distinct variants.

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VASCULAR ENDOTHELIAL GROWTH FACTOR-C AND ITS RECEPTOR TYPE-3 WERE EXPRESSED IN TWO CASES OF ACUTE MYELOGENOUS LEUKEMIA WITH T(3;21)(Q26;Q22)

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Background and *Aims.* t(3;21)(q26;q22) is a recurrent chromosomal translocation in acute myelogenous leukemia (AML), in which the fusion molecule of Runx1 and Evi1 plays an important role in leukemogenesis. This chimeric molecule has reported to up-regulate vascular endothelial growth factor-receptor (VEGFR) type-1 and -2. On some cases of AML VEGF-A acts as an autocrine stimulator for the leukemia blast that expresses VEGFR-1 and -2; however, on our knowledge there have been no reports on the relationship of AML and VEGF-C system. VEGF-C and VEGFR-3 play an important role in the formation of systematic lymphatic vessels. They are also reported to work on progression of malignant solid tumor. We analyzed AML blasts with t(3;21)(q26;q22) in point of VEGF-C system. *Materials and Methods.* Blood cells were obtained from two informed AML patients with t(3;21)(q26;q22) as well as other AML patients, whose mononuclear cells were prepared with density-gradient sedimentation method. Cells were cultured short term for the elimination of an adherent cell-fraction. RNA was extracted from the prepared non-adherent mononuclear cells, and the expression of VEGF-C and VEGFR-3 was analyzed with reverse transcription-polymerase chain reaction (RT-PCR). On the protein level, VEGFR-3 was analyzed with fluorescent activated cell sorter, and VEGF-C levels in patients' sera and in the conditioned media from the cultured AML blasts were determined with ELISA kit (R and D Systems, USA). A growth-inhibition effect by the administration of anti-VEGF-C-neutralizing antibody (Sigma, USA) was assayed with Cell-Counting Kit (Dojindo, Japan). *Results and Discussion*. In two cases of t(3;21)(q26;q22), VEGF-C production was observed at RT-PCR level and the protein level. VEGFR-3 was also expressed in two cases, but not other kinds of AML including RUNX1-MTG8 translocation. When anti-VEGF-C-neutralizing antibody was added to the blast cell-cultures of these two patients, the cell-growth was significantly inhibited. These observations indicate that VEGF-C-autocrine system works on proliferation in AML with t(3;21)(q26;q22).

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ASSESSMENT OF SERUM VARIATION IN ANTI HSP70 ANTIBODIES DURING ACUTE MYELOID LEUKEMIA INDUCTION THERAPY

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Background. Heat shock proteins (HSPs) are overexpressed in many kinds of cancers and are implicated in tumor cell proliferation, differentiation, invasion, metastasis, death, and recognition by the immune system. Although HSP levels are not informative at the diagnostic level, they are useful biomarkers for carcinogenesis in some tissues and signal of differentiation and the aggressiveness of some cancers. Furthermore, the reduction in protein expression levels was correlated with an increased susceptibility to drug-induced apoptosis. HSPs were shown highly expressed by acute myeloid leukemia (AML) cells as well as by acute lymphoblastic leukemia (ALL) cells and HSP expressions were correlated with that of differentiation antigens and that of drug-resistance and apoptosis proteins. Additionally lower expression of HSPs is connected with higher rate of complete remission and significantly longer overall survival. *Aim.* The aim of our study was the assessment of the anti Hsp70 antibody concentration in the patients with acute myeloid leukemia before and during the induction therapy. *Material and Methods.* We assessed 25 peripheral blood samples from the patients with newly diagnosed AML (aged 45-56), including 14 males and 11 females. A group of 10 healthy age matched subjects were used as a control group. The patients underwent the seven day induction therapy (cytarabine, doxorubicin, cladribine). The specimens were obtained before, and 7, 14, 21 days after start of the treatment. Quantitative determination of anti-human Hsp70 antibodies in the serum was done using commercial test (anti Hsp70 Elisa Kits, Stressgen). The results are presented as mean±SEM. Statistical analysis was done using Shapiro-Wilk, Mann-Whitney and Spearman's tests. *Results.* There were no significant difference of baseline anti Hsp70 concentration between AML patients and healthy group (276,3±53,3ng/mL and 236,5±61,4ng/mL

respectively). Furthermore no significant changes in the level of anti Hsp70 antibodies were observed during the treatment. In our analysis the parameters such as age, gender and some leukemic features (as leukocytosis, blastosis in bone marrow and peripheral blood) or remission status after induction therapy have no impact on the assessed antibodies. *Conclusions.* Our study revealed no significant variation in the patient antibody level during induction therapy. At that moment there is no significant correlation between the change of the level of anti Hsp70 before and during the induction therapy to complete remission (CR) or overall survival (OS) but the study regards more patients and longer time of observation.

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NUCLEOSTEMIN GENE EXPRESSION IN ACUTE PROMYELOCYTIC LEUKEMIA PATIENTS

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Background. Nucleostemin (NS), a novel p53-binding protein has been shown essential for stem and cancer cell proliferation and implicated in oncogenesis. Nucleostemin expression had been shown in gastric cancer (SGC-7901) cells, human hepatocarcinoma (HepG2) cells, human cervical cancer (Hela) cells, human osteosarcoma (OS-732) cells. *Aim.* This work designed to study the NS gene expression in bone marrow cells in acute promyelocytic leukemia (APL) patients and in normal bone marrow specimens. *Materials and Methods.* We examined NS gene expression by Quantitative Real Time PCR in bone marrow specimens of 15 cases of APL patients, before treatment and in 4 bone marrow specimens of healthy donors of bone marrow transplantation. In the same samples of bone marrow aspiration morphology of smears was evaluated. Diagnosis of APL was based on morphology and positive PML/RARA in PCR. RT-PCR used to amplify the NS mRNA, and the GAPDH primer sets used for normalizing .For comparison of NS gene expreesion in 2 groups Mann-Whitney U test was used. *Results.* 15 patients enrolled in this study,11(73%) newly diagnosed APL and 4(27%) relapsed cases. Mean age of patients was 28.67±9.56 year. NS gene expressed in all bone marrow samples of APL patients. NS gene expressed in normal bone marrow specimens too. NS gene expression in bone marrow of APL patients was significantly higher than normal bone marrows(P=0.002) Figure 1. There was no significant difference in NS gene expression between newly diagnosed and relapsed APL cases. *Conclusion.* According to the results of this study it seems that NS gene expressed in normal marrow. NS expression in adult bone marrow hematopoietic stem cells had been reported in previous reports and it had been shown that NS does not express in granulocytes and B lymphocytes. It seems that stem cells and proliferating cells in the normal marrow are the source of NS expression detected in normal marrow. NS expreesion in bone marrow of APL patients was significantly higher than normal marrow. In these patients before treatment marrow is replaced by undifferentiated blasts and promyelocytes. We concluded that NS expression in these cells were high.

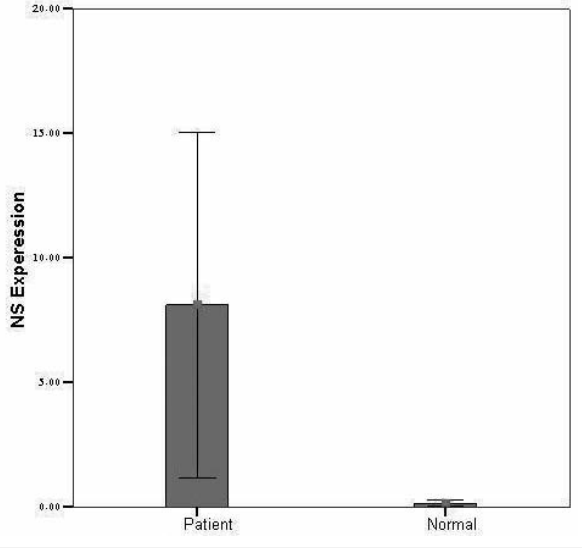

Figure 1. NS gene expression an normal and APL bone marrow.

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CYTOKINE PROFILES IN ACUTE LEUKEMIA PATIENTS AT A TIME OF DIAGNOSIS

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Background. As we know, leukemic cells loose the ability to realize the programming cells death - apoptosis. Cytokines produced by healthy immunocompetent cells, blasts and micro environment's cells, consider being an activators and inhibitors of such process. Insufficient knowledge on the role of cytokine's balance (cytokine profile) in acute myeloid leukemia's process formation and following complications of the decease caused our investigation. *Materials and Methods.* 36 acute myeloid leukemia patients (man - 17, woman - 19) were observed in first acute period. According to FAB classification, patients were grouped as follows: M2 - 2 patients, M4 - 24, M5 - 10. All the patients were of 16-65 years. Before chemotherapy, the plasma samples from the patients were collected for cytokine profile analysis: tumor necrosis factor-α (TNFα) and interleukins (IL) - IL-1, IL-2, IL-4, IL-6, IL-8 and IL-10. The reference range of cytokine concentration in plasma samples considered to be: TNF_ - 5,00-30,00 pg/mL, IL-1 - 10,00-50,00 pg/mL, IL-2 - 35,00-190,00 pg/mL, IL-4 - 1,00-35,00 pg/mL, IL-6 - 5,00-50,00 pg/mL, IL-8 - 0-20,00 pg/mL and IL-10 - 1,00-45,00 pg/mL. *Results.* Concentration of IL-2 in plasma of 23 (64%) patients was within the reference level, 39,210±9,14 pg/mL average, but in 13 patients (36%) we observed remarkable decreasing within this parameter - from 0,01 to 7,250 pg/mL. Level of IL-1 (the average level 7.66±3.04 pg/mL in 86% of patients) slightly decreased. No significant changes were disclosed in IL-4 concentration, its average level was 20.550±9.9; however, this parameter's variation was large within the whole group - from 0.1 to186.0 pg/mL. IL-6 increased the most, comparing to the reference range. Being in average 65.74±11.39 pg/mL, it was higher than normal in 4-5 times in some individual cases. IL-8 level was measured in range from 0.01 to 173.20 pg/mL (in average16.570±5.25 pg/mL), but in 50% cases it increased slightly. Investigation of IL-10 didn't show any difference as within the whole group, as comparing to the reference range (average level was 8.560±0.98 pg/mL). Analyzing of TNFα didn't reveal any remarkable deviations; the average level was 5.56±1.97 pg/mL, maximum range of the measured parameter - from 0.10 to 50.77 pg/mL. *Conclusion.* Thereby, our investigation of the cytokine profile in acute myeloid leukemia patients demonstrated the significant increasing in IL-6 level and a trend toward the decreasing in IL-2 and IL-1 levels in most individuals. Further clarifying of these findings is a focus of our future research.

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THE EFFECT OF ARSENIC TRIOXIDE TREATMENT ON GENE EXPRESSION OF ACUTE PROMYELOCYTIC LEUKEMIA CELL LINE, NB-4

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Background. APL is the most malignant form of acute leukemia with a fatal course of only weeks which represents 10-15% of AML in adults. Arsenic trioxide as a single agent factor (without chemotherapy) is the treatment of choice for APL patients, but the mechanism by which it induces apoptosis remains poorly understood. *Aims.* Since in Iran arsenic is used as first line treatment, it is worth to investigate its effect on expression of genes involved in apoptosis. *Methods.* In this study, we used the human leukemia (NB4) cell line as a model to evaluate the cytoxicity of arsenic trioxide in APL based on the MTT assay. To study the underlying mechanisms of cell death induction by arsenic, we treated NB4 cell line in a time and dose dependent manner. Extracting RNA and synthesis of cDNA, gene expression of apoptotic genes in mitochondrial pathway including caspase3, Mcl-1 and Bcl-2 was analyzed through Real-Time PCR. *Results.* Data obtained from the MTT assay indicated that arsenic trioxide significantly reduced the viability of NB4 cells and inhibited cell growth with a special emphasis on time- and dose-response relationships. We also found that As2O3-induced cell death was paralleled by reduced expression of the antiapoptotic protein BCL2 in both time and dose dependent treatment, but the expression of Caspase3 and Mcl-1 did not change after arsenic treatment. *Conclusions.* These results suggest that changes in BCL2 gene expression may

be one of the mechanisms of action of arsenic through the induction of apoptosis, while Caspase3 and Mcl-1 are not involved in arsenic induced apoptosis.

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P27KIP1 IN ACUTE MYELOID LEUKEMIA

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Acute leukemia (AL) is clonal proliferation of immature leukemia cells in bone marrow (BM), peripheral blood and other tissue. Uncontrolled clonal cell proliferation caused by malignant transformation of cells is a result of many alterations of cell functions involved in cell proliferation, differentiation and apoptosis. Malignant transformation of leukemic cells is also associated with altered activity of cyclin dependent kinases (CDKs) and cyclin-dependent kinase inhibitors CKIs. CDKs form periodically complexes with cyclins and induce cell cycle progression from G1 to S phase. One of CKIs is p27Kip1 member of KIP (kinase inhibitor proteins) family. Aim of this study was to analyze p27Kip1 immunocytochemical expression of BM hematopoietic cells (HCs) in AML patients before cytostatic therapy and in control group of patients. *Methods.* microscopic analysis of p27Kip1 positive HCs was done after immunocytochemical APAAP staining of BM smears. Analyses were done in 14 AML patients and 11 patients of control group (not suffering of hematological malignant disease). BM analysis in control group was done in course of standard diagnostics and from all patients in both groups informed consent was obtained. *Results.* median (8%) and upper limit (45%) of percentages of p27Kip1 positive HCs in control group were higher than same parameters in AML leukemic cells (median: 2,3%; upper limit 18%). Moreover, percentages of p27Kip1 positive HCs in control group were significantly higher (P<0.001) than in AML patients. In 2 out of 14 AML patients was found higher percentage of p27Kip1 positive HCs (5%, 18%). *Conclusions.* Observations are in relation with known p27Kip1 function acting as CKIs of cell cycle progression and with other studies findings implicating variability of p27Kip1 immunoexpression in leukemic cells.

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MYELODYSPLASTIC SYNDROME/ACUTE MYELOID LEUKEMIA IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA TREATED WITH FLUDARABINE

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Aim. Patients with chronic lymphocytic leukemia (CLL) may have disease transformation to diffuse high grade non-Hodgkin's lymphoma (known as Richter's syndroma) or prolymphocytic leukemia. The development of therapy related myelodysplastic syndrome and therapy related acute myeloid leukemia (t-AML/MDS) is unusual. We report the occurence of acute myeloid leukemia (t-AML) and refractory anaemia with excess of blasts (t-MDS/RAEB2) soon after treatement with fludarabine.

Table 1. Laboratory data.

	Age y / gender	Latent Period mo	Therapy for CLL	FAB Typ+ MDS/ AML	Cytogenetic	Therapy MDS/ AML	Survival Mo
1	71/M	24	FC(6 cycles)	M5	46,xy,t(3,7)(q26;q21)[23]	Chemo	1
2	54/M	10	CHOP, FMD, FC	M2	46,xy,del 5(q23;q33),-18,+mar(cp2)/46xy,del(5)(q23,q33),-11,-18,+3mar[cp2]/45xy,del(5)(q23;q33),-11,-18,-21,+3mar [14]/46,xy	Chemo	2
3	69/M	5	FC	RAEB2	46,xy,del(5)(q13,q33),t(11;17)(q21;q25),-20,+mar [13]/46,xy [2]	support	8
4	49/F	14	CHOP, FC	M4	43-46,XX,-5,-13,-18,+3mar [cp20]	chemo	4

Material and Results. In the period from october 2004 to january 2010 a total of 210 patients (pts) with CLL have been treated with Fludarabine. In the same group we have diagnosed t-AML/MDS in 4 pts (1.9%), with median period of latency of 14 months after treatment with fludarabin. The initial CLL treatment was FC protocol (fludarabine 25 mg/m^2 on days 1 to 3, and 200 mg/m^2 cyclophosphamide on days 1 to 3,) in two patients, CHOP and FC protocol in 2 patients (cyclophosphamide 750 mg/m^2, doxorubicin 50 mg/m^2 i.v. and vincristine 2 mg intravenously on day 1and prednisone 100 mg orally given on days 1 to 5.) and in 1 patient in addition to CHOP, FC and FMD protocol was applied (fludarabine 25 mg/m^2 days 1-3, mitoxantrone 8mg/m^2 day 1, and dexamethasone 20 mg/) There were 3 males, 1 female, with age ranging from 49 to 71 years. According to FAB classification of acute myeloid leukemias there was 1 pts of M5b type, 1 case of M4, 1 case of M2 and 1 of RAEB2 with 17% of blastas in bone marrow. The characteristics of patients at the time of presentation are summarized in Table 1. All pts had cytogenetic abnoramlities commonly seen in therapy related AML, complex karyotype and abnormalities of chromosome 5 in three patients. One patinet had t(3;7)(q29;q21). Three patients were treated with reduced doses of doxorubicin and cytosin/ arabinoside and one patient with RAEB2 with supportive therapy. The patient with M4 achieved a partial response but after second course of chemotherapy developed enterocolitis, ileus and after surgery died. The best survival was in patient with RAEB treated with supportive therapy (8 months). The other three patients survived 2 m, 1 and 4 months respectively. *Conclusion.* Second malignancies occur with increased frequency in patients with CLL, but they may be more frequent and more aggressive after nucleoside analog therapy of CLL.This report may suggest that fludarabin might predispose to secondary malignancies but this preposition needs further evaluation by long term studies.

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LACK OF PROGNOSTIC IMPACT OF WT1 MUTATIONS IN NORMAL KARYOTYPE ACUTE MYELOID LEUKEMIA: RESULTS OF THE LAM2003+G-CSF PROTOCOL OF THE CETLAM STUDY GROUP

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Background. Wilms tumor 1 (WT1) is a transcription factor which is commonly up regulated in patients with Acute Myeloid Leukemia (AML). Acquired mutations of the WT1 gene have been reported in approximately 10% of normal karyotype (NK) AML and have been associated with a bad outcome. *Methods.* Samples from 165 adult patients with *de novo* NK-AML and included in the LAM2003+G-CSF protocol were analyzed for WT1 mutations. Capillary electrophoresis for exon 7 and direct sequencing for exon 9 were performed for each case using an ABIPRISM 310 Genetic analyzer. NPM1, FLT3-ITD, CEBPA and MLL status was also investigated using well established protocols. WT1 mRNA levels were studied by means real-time PCR following the primers and conditions described by the LeukemiaNet group.*Results.* WT1 mutations were identified in 19 patients (11.5%) and were significantly associated with the presence of CEBPA mutations (P=.021). There was no significant association with NPM (P=.220), FLT3-ITD (P=.449) and MLL (P=.614) mutations. Subset analysis according to the FLT3-ITD status did not reveal any significant impact. Complete remission (CR) rates were not significantly different between patients with WT1 mutations and those with unmutated WT1 (P = .40, 84.2% v 71.9%). There was no difference in disease free survival (DFS; P= .887; 3-year rates, 52.4% v 48%), in overall survival (OS; P=.445; 3 years rate, 40% v 36%) and in refractory disease (P=.705). Patients with WT1 mutations show a trend to statistical significance with regard to the G-CSF use (P=.090). Furthermore, G-CSF use had a favorable impact on OS (P=.013; 3 years-rate, 69% v 30%) (P=.090) and an inverse correlation trend with induction failure (P=.069). *Conclusions.* WT1 mutations were devoid of adverse prognostic meaning in this series of NK-AML. WT1 mutations were significantly associated with CEBPA mutations. G-CSF treatment may play a protective role in patients with WT1 mutations.

1238

CYTARABINE AND AMSACRINE FOR AML SALVAGE THERAPY IN ELDERLY PATIENTS: A SINGLE CENTER RETROSPECTIVE STUDY

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Hopital Haut Leveque, Pessac, France. *In elderly patients with acute myeloid leukemia (AML) treated intensively, there are little data supporting the efficacy of salvage chemotherapy in patients with refractory or relapse AML*

We performed a retrospective study in 42 patients over 55 years old with the aim to investigate prognostic factors for survival. Salvage chemotherapy consisted in the association of cytarabine 3 g/m_/12 hours D1-D3 (total 5 doses) and amsacrine 150 mg/m^2/day D1-D5. The median age was 64 years (range 58-74). Of these 42 patients, 10 (23.8%) had refractory AML, 18 (42.9%) had a <12 month relapse and 14 (33.3%) a >12 month relapse. There were 3 (7.1%) AML with favorable cytogenetic risk, 29 (69%) AML with intermediate cytogenetic risk and 9 (21.4%) AML with unfavorable cytogenetic risk. Seven patients (16.7%) achieved a complete response (CR), 4 patients (9.5%) achieved a complete response with incomplete blood count recovery (CRi), and 4 patients (9.5%) achieved a partial response (PR). Twenty five patients (59.6%) had a refractory AML, and two patients died within 30 days after D1 chemotherapy. The median time until neutrophil recovery (> 0.5×10^9/L) was 20 days. Febrile neutropenia was universal with 47.6% grade III-IV infection toxicity, 38.1% septicemia and 16.7% invasive fungal infection. After a median follow-up of 4.2 months (range 24 days-7.8 years), median overall survival (OS) was 5 months (range 24 days-7.8 years), median event free survival (EFS) was 51 days (range 18 days-6.8 years) and median relapse free survival (RFS) was 5.9 months (range 31 days-6.7 years). Among 11 patients who achieved CR or CRi, 4 (36.4%) received a consolidation with chemotherapy alone including high-dose cytarabine, 2 patients (18.2%) underwent autologous hematopoietic stem-cell transplantation (HSCT) and 3 (27.3%) allogeneic HSCT. Among 4 patients who achieved PR, 3 received an allograft. Among 8 (19%) patients who underwent HSCT, there were 1 AML with unfavorable cytogenetic risk, 5 AML with intermediate cytogenetic risk and 2 AML with favorable cytogenetic risk. Median OS was 16.5 month (range 6.8 month-7.8 years) for patients who proceeded to transplantation *vs.* 3.5 months (range 24 days-3.3 years) for patients who did not. Median EFS was 5 month (range 1.2 month-6.8 years) for patients who proceeded to transplantation *vs.* 47 days (range 18 days-3.2 years) for patients who did not. In univariate analysis, there is evidence that autologous and allogeneic HSCT (respectively P=0.02 and P=0.0025) could positively affect overall survival among patients over 55 years old. In multivariate analysis, two good prognostic factors affected overall survival: non unfavorable cytogenetic risk evaluated at diagnosis (P=0.02) and consolidation treatment by chemotherapy or HSCT (P<0.001). Our findings suggest that cytarabine-amsacrine offers a reasonnable salvage chemotherapy and that consolidation treatment by HSCT is necessary to sustain the response.

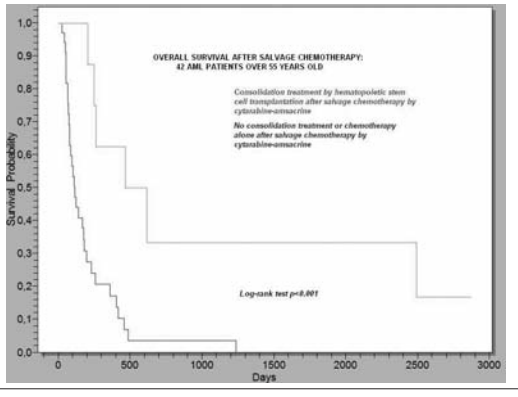

Figure.

1239

EVALUATION OF PLASMA CYTOKINE LEVELS BY BIOCHIP ARRAY TECHNOLOGY IN ACUTE MYELOID LEUKEMIA PATIENTS. A PILOT STUDY

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Background. Cytokines and growth factors have been studied as markers of immune system activation in various diseases including hematological malignancies. *Aims.* The aim of our pilot study was to determine plasma levels of multiple cytokines by biochip array technology in acute myeloid leukemia (AML) patients. *Methods.* A total of 15 AML patients (mean age 48.7±12.1 years, median 51.0, 8 males and 7 females) treated with cyclic chemotherapy (3+7, 2+5, HiDAC) alone or in combination with high-dose chemotherapy (preparative regimen Bu/Cy2 or Cy/TBI) followed by autologous hematopoietic cell transplantation were studied. We evaluated plasma levels of the following cytokines: interleukin-1 alpha (IL-1 alpha), interleukin-1 beta (IL-1 beta), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), epidermal growth factor (EGF), monocyte chemotactic protein-1 (MCP-1). All biomarkers were measured by biochip array technology on Evidence Randox analyzer at the diagnosis of AML (active leukemia) and at 6 months after completion of chemotherapy (durable complete remission in all patients). *Results.* Comparing cytokines levels in active leukemia and in durable complete remission, we found significant decrease in plasma IL-1 β (2.56±3.27 ng/L *vs.* 1.63±2.17 ng/L; P<0.05), IL-6 (46.24±83.14 ng/L *vs.* 2.49±2.51 ng/L; p < 0.05), IL-8 (104.99±167.30 ng/L *vs.* 11.72±4.34 ng/L; p < 0.05), IL-10 (7.58±14.15 ng/L *vs.* 2.22±4.78 ng/L; P<0.05) and TNF-α (4.65±4.27 ng/L *vs.* 2.19±1.13 ng/L; P<0.05). On the other hand, we found significant increase in VEGF (63.93±67.85 ng/L *vs.* 114.39±54.90 ng/L; p < 0.01) and EGF (16.48±33.50 ng/L *vs.* 64.42±35.33 ng/L; P<0.001). Plasma levels of other cytokines (IL-1 α, IL-2, IL-4, INF-γ, MCP-1) were without significant differences. *Conclusions.* Our results indicate that plasma levels of some cytokines and growth factors (EGF, VEGF, IL-1 β, IL-6, IL-10, TNF-α) could serve as useful diagnostic and prognostic parameters for AML patients, showing activity of the disease. Further studies in a larger number of AML patients and comparing cytokine levels with healthy subjects will be needed to define the potential role of these and additional biomarkers in this context.

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THE OUTCOME OF ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION FOR PRIMARY REFRACTORY OR RELAPSED ACUTE MYELOID LEUKEMIA

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Background. The outcomes of patients with primary refractory or relapsed after chemotherapy are dismal. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) provides curability for those cases through graft-versus-leukemia effect. *Aims and Methods*. The data of 40 primary refractory to induction chemotherapy and 32 relapsed cases were retrospectively reviewed to investigate the clinical factors that determine the long-term outcomes of allo-HSCT in those cases. *Results.* Primary engraftment failure and veno-occlusive disease occurred in 9 (12.7%) and 12 (16.7%) patients. The 3-year cumulative incidence of chronic graft-versus-host disease (GVHD) and extensive chronic GVHD was 36.0% and 23.5%, respectively. With a median follow-up duration of 5.2 months (range, 0.1-129.4), 5-year overall survival (OS) and disease free survival (DFS) were 18.4% and 14.8%. The causes of death were relapsed-related in 23, GVHD in 10, and infection in 18 patients.

The 5-year cumulative incidence of relapse was 43.0%. The 5-year OS was higher for the patients with chronic GVHD (26.2%) than for those without chronic GVHD (9.0%; P<0.001). Plus, the 5-year OS rate was not significantly different between high WBC index (≥20) and low WBC index (<20) groups (WBC index = WBC (109/L) x [% of marrow blasts/100] at the time of transplantation). In the multivariate analysis, the only variable associated with higher OS was the presence of chronic GVHD (hazard ratio 0.253, 95% confidence interval 0.110-0.582; P<0.001). *Conclusion.* In conclusion, the transplantation outcome of primary refractory or relapsed AML was poor. However, the presence of chronic GVHD indicated favorable outcome in terms of overall survival.

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ACUTE PROMYELOCYTIC LEUKEMIA (APL) IN VERY ELDERLY PATIENTS (> 70 YEARS)

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Background. Acute Promyelocytic Leukemia (APL) is a rare subtype of Acute Myeloid Leukemia (AML) which is more common in younger adults, with a median age of 45-50 years at onset. *Aim and Methods.* The use of All-trans Retinoic Acid (ATRA) as tailored treatment has made APL a very curable disease also in patients aged > 60 years; however, there are only few case reports in very elderly APL patients. To address this issue, we revised clinical data and treatment results in 12 patients aged > 70 years with newly diagnosed APL followed at our Institution from 1/91 to 12/2008. *Results.* Clinical characteristics at onset were as follows: M/F 7/5, median age 74.7 years (range 70.0-80.8), M3/M3v 11/1, median WBC 1.3×10^9/L (range 1.0-7.4), median PLTS 53×10^9/L (range 12-302), BCR1/BCR3 6/6. According to Sanz risk score, 7 patients were at low-risk and 5 at intermediate-risk; 6/12 patients had arterial hypertension, 4/12 a concomitant cardiologic disease, 3/12 a cerebrovascular disease and 2/12 a previous neoplasia. Induction therapy consisted of ATRA + Idarubicin in 8 patients (2/8 with reduced Idarubicin dosage) and ATRA alone in 4 patients; in this latter group, however, 2/4 needed to add chemotherapy (CHT) based on Mitoxantrone + AraC due to hyperleukocytosis during ATRA treatment. All patients achieved both morphological and molecular Complete Remission (CR) after a median time of 50 (range 29 - 65) and 105 (range 51 - 239) days, respectively. Infective complications were observed in 10/12 patients (4 episodes of FUO, 6 sepsis, 2 cystitis and 1 oral abscess) while ATRA syndrome occurred in 2/12 patients; in addition, there were 3 episodes of cardiac ischemia and 3 episodes of paroxystic atrial fibrillation. All but one patient received consolidation therapy (based on CHT alone in 7 patients, CHT + ATRA in 3 patients and ATRA alone in 1 patient), followed by maintenance treatment in 8 patients. Four patients had a relapse (hematological in 3 cases and molecular in 1 case) after 8, 11, 35 and 56 months respectively. At present, 6 patients are still alive, 4 died due to disease progression (3) or senectus while in CR (1) and 2 were lost to follow-up while in CR: mean event-free survival and overall survival were 89.2 months (95%CI 52.6 - 125.8) and 99.9 months (95%CI 65.0-134.7), respectively. *Conclusions.* ATRA-based treatment of APL is safe and effective also in very elderly patients, with long-lasting disease-free and overall survival.

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INCIDENCE, CLINICAL CHARACTERISTICS AND OUTCOME OF ACUTE MYELOID LEUKEMIA PRESENTING WITH CONCURRENT EXTRAMEDULLARY LEUKEMIC TUMORS: A SINGLE-CENTRE EXPERIENCE

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Introduction. Acute myeloid leukaemia with extramedullary leukemia (EML) at presentation has been the object of few studies. Most of publications concern case reports. Data about incidence and prognostic impact are variable and conflicting and probably reflect lack of definite criteria. *Materials and Methods.* We examined retrospectively 214 consecutive adults up to 60 years of age with *de novo* AML, admitted to our institution from 1995 to 2009 and uniformly treated. The median age was 45 years (range16-59). There were 103 male and 111 female. Median follow-up was 19,3 months. We found 14 patients (6,5%) with concurrent EML at presentation. Outcome of this subset of patients was compared with patients without extramedullary involvement. *Results*. There were 6 male and 8 female. The median age was 40 years. The anatomical sites affected were: skin (n=4), skin and cervical lymph nodes (n=1), spleen (n=1), central nervous system (n=2), ovary (n=1), parotid gland (n=1), abdominal lymph nodes and kidneys (n=1), cervical lymph nodes (n=1), paraspinal (n=1). Cytologic or hystologic diagnosis of extramedullary localizations were performed in all patients except four (the patients with central nervous system and paraspinal localizations and the patient with skin and cervical lymph nodes involvement). Median of white blood cells (WBC) count at presentation was 83×10^9/L (range 1,3 -180). Median lactate dehydrogenase (LDH) level was 790 U/L. According to FAB classification there were 2 M0, 4 M1, 1 M2, 5 M4, 2 M5. Cytogenetic analysis was performed in 13 patients. They were categorized according to Medical Research Council criteria. Two patients had favourable karyotype and 11 patients had intermediate karyotype (9 patients with normal karyotype and 2 patients with abnormality 11q23/MLL). Data about FLT3 mutations were available for 13 patients: there were 3 FLT3-ITD mutations (23%) and 1 FL-TKD mutation (7%). Age, WBC count, FAB subtype, karyotype, FLT3 mutation did not differ statistically in the two populations. Complete response rate was 50% in patients with EML and 63% in patients without EML (P=0.113). Relapse free survival (RFS) did not significantly differ in the two populations: 14,4 months in the EML group *vs.* 16,1 months (P=0.869). Overall survival (OS) did not significantly differ in the two populations: 19,4 months in the EML group *vs.* 23,5 months (P=0.931). In a Cox multivariate analysis, both OS and RFS were not influenced by presence or absence of extramedullary involvement, even when adjusting by gender, FAB, FLT3, WBC count and karyotype. *Conclusions.* Incidence of AML with EML in our institution was 6,5%. Skin was involved alone or with other tissues in 35% of patients. Fifty per cent of patients had FAB M4 or M5. A normal karyotype was found in 69% of cases. Rate of FLT3 mutations did not differ from that in literature. We did not find a statistical difference in the outcome of this subset of AML.

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RELATION BETWEEN PHENOTYPIC FEATURES AND MOLECULAR PROGNOSTIC MARKERS IN ADULT ACUTE MYELOID LEUKEMIA

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Background. The FLT3 gene is frequently mutated in acute myeloid leukemia (AML), either by internal tandem duplication (ITD) of the juxta-membrane domain or by activating point mutations in the second tyrosine kinase domain (TKD). Together with nucleophosmin (NPM1) gene mutation, FLT3 alterations are the most frequent molecular changes in AML. FLT3-ITD is related to a poor prognosis of the patients. Mutations in exon 12 of the NPM1 gene are the most frequent genetic abnormality in adult AML, found in 50-60% of patients with normal karyotype and are associated with a better prognosis. Interestingly, this prognostic advantage of NPM1 mutations can only be observed in patients lacking additional FLT3-ITD mutation. *Aims.* We have analyzed the correlations of FLT3 and NPM1 mutations with phenotypic features and karyotype in adult patients with AML diagnosed at our Institution between 2006 and 2009. *Methods.* Diagnosis of the patients was made by bone marrow cytology and karyotype, and classified according to the WHO criteria. Immunophenotyping was made using three-color combinations of monoclonal antibodies. Genomic DNA was extracted by phenol-chloroform. Genotyping was made with the MegaBACE 1000 equipment and analyzed by the software Fragment Profiler v1.2. For detection of the FLT3-TKD mutation, genomic DNA was amplified by PCR followed by restriction analysis. Methylation of p15 and p73 were also analyzed (COBRA and MSP techniques respectively). *Results.* We studied 103 cases. Median age: 52 years (15-97). PB leukocytes: 24.8×10^9/L. In 71 cases the karyotype was available: 15 had t(15;17), 6 had an otherwise low risk karyotype, 33 had a normal, and 17 a high risk karyotype. NPM1 was mutated in 25 cases. All except one had a normal karyotype. However, 44% presented FLT3-ITD. Patients with methylated p15 had a higher frequency of FLT3-TKD and higher leukocyte counts. Those with methylated p73 had a lower frequency of FLT3-ITD as well as lower peripheral leukocytes. Cases without any of the mutations studied had higher hemoglobin (P=0.023) and lower leukocyte counts (P=0.0008). The cases with unmutated NPM1 had lower expression of CD11b (P=0.006) and CD33 (0.028) and a higher expression of CD34 (P=0.01). Patients with FLT3-TKD mutation were

older (P=0.017) and had a higher expression of CD33 (P=0.043). *Conclusions.* Among our patients, NPM1 mutation, although occurring in patients with a normal karyotype, was associated with FLT3-ITD. Besides, in our study there was a high frequency of high risk karyotype. Therefore, several factors influenced the prognosis of the patients. Concerning phenotypic features, the patients with mutated NPM1 had a more mature phenotype. These findings could have important implications for the therapeutic use of anti-CD33 in patients with newly diagnosed AML.

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SERUM FERRITIN AS A PROGNOSTIC MARKER IN YOUNG ADULT ACUTE MYELOID LEUKEMIA

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Background. Different studies have demonstrated an iron overload contribution to post-transplantation liver toxicity, infectious events and poor survival in patients undergoing hematopoietic stem cell transplantation for haematological malignancies. *Aims.* So far, in the clinical setting of adult acute myeloid leukaemia (AML) there is no evidence of the possible role of iron in response and survival rates. We studied the role as a prognostic factor of pre-treatment serum ferritin in young adult AML. *Methods.* The study sample included 43 consecutive adult *de novo* AML patients (17 males and 26 females, median age 46 years, range 16 to 60 yrs). The serum ferritin level was determined at onset of the disease in each case. According to the FAB criteria the subtypes were: 3 M0, 29 M2, 6 M4, 4 M5, 1 M6. M3 subtypes were excluded from the analysis. NPM, FLT3 and cytogenetic evaluation was performed for all cases. The NPM mutation was present in 16 patients (37%) and 16 (37%) harboured the FLT3 alteration (ITD: 9 (21%); D835: 7 (16%)). Sixteen (37%) patients were in the unfavourable cytogenetic group, 8 (19%) in the favourable group and 19 (44%) presented a normal karyotype (NK). The patients were subdivided into two groups according to serum ferritin values (<800 *vs.* > 800 ng/mL). Student's t-test or the Mann-Whitney test was performed for comparisons of means. Two-tailed Fisher's exact test was used to compare categories. Overall survival (OS) was measured from the time of diagnosis to death or last follow-up visit and was calculated using the Kaplan-Meier method; the log-rank test was used to compare survival curves. Logistic regression was performed for multivariate analysis. Only p values <0.05 were considered statistically significant. *Results.* Seventeen (40%) patients showed a ferritin serum value > 800 ng/mL. Compared with the <800 ng/mL group, patients with serum ferritin > 800 ng/mL were more frequently non responders to chemotherapy (35 *vs.* 73%, P=0.003) and they had a shorter OS (235 *vs.* 657 days, P=0.006). Moreover, patients with serum ferritin >800 ng/mL showed a higher frequency of documented infections during induction treatment (35% *vs.* 4%, P=0.001). At multivariate analysis, FLT3, NPM, Cytogenetic and Ferritin value (<o>800 ng/mL) all showed a statistical correlation with the response rate (P=0.02; P=0.05; P=0.03; P=0.03, respectively). *Conclusions.* The results of our study suggest a link between serum ferritin and AML prognosis. Further studies are needed to confirm the utility of serum ferritin as a prognostic marker in the adult acute myeloid leukaemia setting.

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THE EXPRESSION OF BMPS RECEPTORS CORRELATES WITH RESPONSE TO INDUCTION THERAPY AND SURVIVAL IN PATIENTS WITH ACUTE LEUKEMIAS

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Background. The pathogenesis of acute leukemias (AL) is related with abnormalities of cells signaling pathways, proliferation and apoptosis. Bone morphogenetic proteins (BMPs) are multifunctional cytokines which belong to the transforming growth factor β (TGFβ) family. They participate in the regulation of growth, differentation and apoptosis in a variety of cell types including hematopoietic lineages. The role of BMPs in pathogenesis of acute leukemias remains unclear. *Aim*. The aim of this study was to evaluate the expression of BMPRIA, BMPRIB and BMPRII on the blast cells and their significance as prognostic factors in patients with acute leukemias. *Materials and Methods.* 70 patients with newly diagnosed AL were evaluated (28 females and 42 males). The median age of patients was 52 years. There were 57 patients (81%) with acute myeloid leukemia (AML) and 13 patients (19%) with acute lymphoblastic leukemia (ALL). The diagnosis was performed according to the FAB criteria for AML patients and EGIL criteria for ALL patients. The healthy control group included 10 age-matched individuals (5 females and 5 males). Bone marrow samples were taken before induction therapy. The mononuclear cells from bone marrow were separated by density-gradient centrifugation. The expression of BMPRIA, BMPRIB and BMPRII was detected by using specific antibodies anti-BMPRIA, anti-BMPRIB and anti-BMPRII (R&D Systems). Leukemic cells were gated based upon their CD45 expression. Two-colour immunofluorescence stainging was performed. The expression of BMPs receptors was analyzed for fluorescence on PAS flow cytometer. The results were statistically analyzed using 'STATISTICA 8,0'. Statistical analysis was performed by means of Mann-Whitney's U-test and P<0,05 indicated a significant difference. The survival of patients was analyzed by the Kaplan-Meier method. *Results*. 38 patients (54%) with AL achieved complete remission (CR) after induction therapy, 6 patients (9%) achieved partial remission (PR) and 26 patients (37%) had no response. The percentage of leukemic cells with expression of BMPRIA, BMPRIB and BMPRII was significant higher in patients with CR than in patients with NR. We observed that expression of BMPRIA, BMPRIB and BMPRII on leukemic cells were significantly lower in patients with early relapse (up to three months after induction therapy) in comparison with patients still being in remission. Patients with the higher percentage of leukemic cells with expression of BMPRIA and BMPRII had the longer survival than patients with the lower BMPRIA and BMPRII expression. The results are shown in Table 1. *Conclusion.* Our results suggest that BMPs receptors could be an independent prognostic factor for response rate and survival after induction therapy in patients with acute leukemias. This observation should be validated by larger study.

Table 1. BMPs receptors and response to treatment.

	CR+PR n=44	NR n=26	Early relapse n=20	Without relapse n=50	p
BMPRIA	39,67±12,51	13,72±6,44	17,54±10,88	27,79±18,27	<0,05
BMPRIB	44,56±7,38	13,45±5,82	19,37±15,35	28,81±17,62	<0,05
BMPRII	44,41±9,62	12,33±3,50	19,58±14,24	28,60±17,89	<0,05

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A SIMPLE MOLECULAR PROFILE AT DIAGNOSIS MAY PREDICT THE PROBABILITY OF ACHIEVING COMPLETE REMISSION IN UNTREATED DE NOVO NON-M3 AML PATIENTS

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Background. FLT3 mutations and expression of NPM, WT1, BAALC and other genes may exert a relevant role in predicting complete remission and outcome of AML patients. *Aims and Methods.* A molecular profile was performed in 93 consecutive untreated non M3 denovo AML patients receiving induction chemotherapy (standard induction in 38 patients, fludarabine-ara C-idarubicin and low dose gemtuzumab ozogamicin in 65 patients) with the aim of predicting CR rate and long term outcome. Expression levels were obtained by Real-Time-PCR upon normalization on Abl expression. *Results.* 51 patients were younger than 60 years (median age 50) and 42 were older (median age 69). Intermediate or favourable karyotype was detected in 73 patients (78%). Unfavorable karyotype was more frequent in patients older than 60 years, compared with younger ones (31% *vs.* 14%, respectively, p 0.04). FLT3: 20 patients (21%) had ITD and 7 (8%) had exon 20 mutations. WT1: in 24 patients (26%) expression was > 2365 (>75th percentile); in 69 < 2365 (74%); NPM : the gene was mutated in 37 patients (40%) and non mutated in 56 (60%); BAALC: in 47 patients expression was <1000 (51%), in 46>1000 (49%). Molecular profile was similar in the younger and in the older subgroups of patients (p NS). In the entire cohort of patients CR rate was not influenced by the type of induction. CR rate was higher in patients aged 59 or less (P<0.001), in patients with normal or intermediate karyotype (not unfavourable karyotype)

(73% compared with 30% in patients with unfavourable karyotype, p 0.001), in patients with mutated NPM gene (76%, compared with 55% in non mutated NPM; p 0.03), in patients with BAALC expression < 1000 (75% compared with 52 in patients with BAALC expression > 1000, P 0.02) and in patients with WT1 expression > 2365 (79% compared with 56% in patients with WT1 expression < 2365; p 0.05). The presence of FLT3 ITD or FLT3 mutations at exon 20 did not affect CR rate. In the group of 73 patients with not unfavourable karyotype the level of WT1 expression retained a statystical influence on CR rate (90% in patients with WT1 expression > 2365, compared with 65% in patients with WT1 expression < 2365; p 0.03), whereas mutation of NPM gene and BAALC expression had a borderline influence on CR rate (p 0.07 and 0.08, respectively). *Conclusions.* In the whole cohort of denovo AML patients age, karyotype and a simple molecular analysis at diagnosis, including BAALC and WT1 expression and mutational status of NPM gene may predict the probability of achieving CR. In the subgroup of patients with not unfavourable karyotype high (> 2365) WT1 expression was the best positive predictor of CR. A longer observation and further statystical analysis are required to evaluate whether this will be associated with an improved outcome also.

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INTERNAL TANDEM DUPLICATION OF FMS-LIKE TYROSINE KINASE-3 DETERMINES A SIGNIFICANT PROGNOSTIC FACTOR ON ACUTE PROMYELOCYTIC LEUKEMIA

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Background and *Aims.* Internal tandem duplication of fms-like tyrosine kinase-3 (FLT3-ITD) is observed in 20-30_ of the newly diagnosed acute myelogenous leukemia (AML), and that shows significant poor-prognosis in AML with normal karyotype on the response to chemotherapy and over-all duration of the survival. On acute promyelocytic leukemia (APL), it has been still under discussion whether FLT3-ITD is a significant prognostic factor. To clarify this point, we observed FLT3-ITD and clinical outcome of APL prospectively. *Patients, materials and Methods.* We analyzed APL patients (pts), who hospitalized to our institute from Nov. 1998 to Dec. 2009. Bone marrow cells were collected from informed patients, and mononuclear cells were separated with gravity-sedimentation method. After elimination of an adherent cell-fraction, RNA was extracted from the prepared non-adherent mononuclear cells. FLT3-ITD was analyzed with reverse transcription-polymerase chain reaction (40 cycles). *Result.* There were 26 pts (median age 46.5, range 18-74 years) with a diagnosis of APL who were screened for FLT3-ITD. Fifteen pts were male, and 11 were female. Of the 26 pts, 6 (23%) presented with FLT3-ITD. A median WBC counts at the time of diagnosis were 215×10^9/L (range 22-1561×10^9/L) and 16.5×10^9/L in FLT3-ITD positive pts and negative ones, respectively. All pts were treated on Japan Adult Leukemia Study Group APL97 protocol. All cases achieved complete remission. The relapse rates of FLT3-ITD positive pts and negative ones were 100% and 5%, respectively. Among FLT3-ITD positive pts, median duration to 1st relapse from diagnosis was 469 days (range 240-737). The rates of CNS involvement were 50% and 5%, respectively. In FLT3-ITD negative pts group, only one pts had experienced relapse. Interestingly, FLT3-ITD was detected in this pts when bone marrow relapse was demonstrated. *Discussion and Conclusion.* FLT3-ITD plays by itself an important prognostic factor for the relapse and CNS involvement on APL. We should consider an intensive treatment strategy to the FLT3-ITD positive APL pts during maintaining 1st CR.

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ACUTE MYELOID LEUKEMIA WITH MYELODYSPLASIA RELATED CHANGE SHOWED A LOWER COMPLETE REMISSION RATE, BUT EQUIVOCAL SURVIVAL COMPARED TO THOSE WITHOUT MYELODYSPLASIA RELATED CHANGE

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Background. Revised World Health Organization (WHO) classification adopted acute myeloid leukemia (AML) with myelodysplasia related change (MRC) as new disease entity. AML with multilineage dysplasia is known to be associated with worse prognosis although it is still in debate. *Aims.* The present study attempted to evaluate the impact of the presence of MRC at the time of diagnosis on the achievement of complete remission (CR), overall (OS) and leukemia free survival (LFS)in patients with AML. *Methods.* The current retrospective study includes AML patients receiving induction chemotherapy between 2001 and 2008 at the Samsung Medical Center, Seoul, Korea, and having available report of marrow morphology data including marrow aspirates and biopsy specimen. According to the presence of MRC in the marrow samples, followings were compared such as patients' demographics, clinical characteristics, CR rate, LFS and OS. *Result.* A total 286 of patients were included, of whom median age was 47 years (15-81). Forty nine patients (20.2%) presented MRC in the marrow samles. The patients with AML-MRC showed characteristics of higher frequency of unfavorable karyotype (P=0.020), and none of them had favorable karyotype. In order to investigate the clinical implication of MRC on CR rate, OS and LFS, 44 patients having favorable karyotype were excluded from the analysis, and finally data of 242 patients with intermediate or unfavorable karyotype were analyzed. Compared to those without MRC, the patients with AML-MRC showed lower WBC counts (P<0.001) and peripheral blast counts (P<0.001), but was not associated with age or gender.. However, those with AML-MRC showed a lower CR rate at 57% compared to those without MRC (79%, P=0.002). Although those without AML-MRC showed higher CR rate, it was not directly translated into better survival: Between those with MRC and without MRC, there was no statistical differences of LFS (P=0.190) or OS (P=0.365). In Cox proportional hazard model, MRC was not identified as an independent prognostic factor for OS (HR 0.952 P=0.834), while an achievement of CR (P<0.001) and cytogenetic risk group (P=0.02) were found to be significant prognostic factors for OS. *Conclusion.* Although CR rate following induction chemotherapy was lower in patients with AML-MRC, they do not seem to have inferior outcomes than those without MRC. It is not necessary to decide different treatment strategy according to the presence of MRC in initial marrow.

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COMBINED EPIGENETIC AND SIGNAL TRANSDUCTION THERAPY MODULATE SPECIFIC SIGNAL TRANSDUCTION PATTERNS IN ACUTE MYELOID LEUKEMIA

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Elderly patients with secondary acute myeloid leukemia (AML) have a limited response to conventional therapy and short survival. The advanced age and comorbidities leads to limited tolerance for intensive chemotherapy. Cell signaling and responses to growth factor stimulation is frequently altered through mutations in key signal nodes of AML. We treated selected elderly AML patients with all-trans retinoic acid (ATRA) for two days alone before adding theophylline and the histone deacetylase inhibitor valproic acid (ClinicalTrials.gov NCT00175812; EudraCT no. 2004-001663-22). Single cell signaling was determined before treatment and during therapy by phospho-flow cytometry in AML cells stimulated with various growth factors and cytokines. A combination of seven different stimuli and phosphorylation of eight signaling proteins (CREB, STAT1/3/5, p38, Erk1/2, Akt and rpS6) indicated perturbations in signal transduction pathways associated with less aggressive growth and differentiation after therapy. AML cellular subsets responded differently to stimuli and were modulated during therapy. Individual signaling profiles together with clinical and hematologic information may identify responders of low toxicity therapy of AML.

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EXPRESSION OF GRP78 AND OCT1 IN ACUTE MYELOBLASTIC LEUKEMIAS

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Background. Despite continuous progress in therapy of AML, relapses and resistance to chemotherapy remain a challenge for the physician.

GRP78/BiP (glucose regulated protein 78/ immunoglobulin heavy-chain binding protein) and OCT1/SLC22A1 (organic cation transporter 1/ solute carrier family 22, member 1) are linked to drug resistance in solid tumors. There are no data about role of expression of these proteins in AML. *Aims.* The main objective of this study was to compare the expression of GRP78 and OCT1 mRNA between non-M3 AML patients and healthy individuals. *Methods.* Using quantitative reverse transcriptase PCR, the mRNA expression of two genes GRP78 and OCT1 was measured. Bone marrow samples of 83 AML patients (median age 49 years, range 19-84) taken at diagnosis, comprising all subtypes on the basis of FAB classification (without M3 subtype) were assessed. The control group consisted of 8 bone marrow aspirates from healthy individuals. The relative quantitation was indicated by cycle threshold (Ct) values. The Ct value of the target genes was normalized (_Ct) to the Ct value of the ABL gene of the samples. *Results.* GRP78 and OCT1 mRNA were expressed in all samples. A statistically significant (P=0.00001) decrease in OCT1 mRNA expression was observed in AML patients compared with control group (ΔCt 7.382 SD 2.11 *vs.* ΔCt 3.06 SD 1.269 respectively). GRP78 mRNA expression was higher in AML samples than in healthy individuals (ΔCt -4.791 SD 0.75 *vs.* ΔCt -5.097 SD 0.453 respectively), although there was no statistically significant difference (P=0.212). There was also no statistical correlation between the expression levels of GRP78 and OCT1 in AML patients and complete remission rate. *Conclusions.* Our preliminary data indicate differences in expression of GRP78 and OCT1 mRNA in AML patients compared to healthy individuals. Further studies should be undertaken to demonstrate their clinical impact in acute myeloblastic leukemia.

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MYELOID SARCOMA: CLINICAL CHARACTERISTICS AND PROGNOSIS

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Introduction. Myeloid sarcoma (MS) is a rare hematologic neoplasm which was defined by WHO classification as a separate myeloid tumor. Any site of the body other than the bone marrow (BM) can be affected by this tumor. MS may occur *de novo* and as the manifestation of relapse in patients (pts) with previously diagnosed acute myeloid leukemia (AML). The aim of the study was to investigate clinical characteristic and prognosis in pts with *de novo* MS. *Methods.* We observed 121 pts with AML treated at N.N. Blokhin Cancer Research Center RAMS from 2000 to 2010. In this study we included pts with *de novo* MS with extramedullary lesions without BM involvement (according to BM smears and BM biopsies examinations). MS diagnosis was based on histological and immunohistochemical tumor mass studies. Immunohistochemical study was performed with monoclonal antibodies for MPO, lysozyme, CD33, CD13, CD117, CD15, CD68, CD34, TdT, PGM1. *Results.* MSs *de novo* were revealed in 8 pts (6,6%) and as relapse of AML in 2 pts (1,7%). Median age of pts with MSs *de novo* was 39 years (range 26-61). Male: female ratio - 3:5. MSs localizations: 3 cases of genital system lesions (2 - vagina and regional lymph nodes, 1 - uterus, ovary and regional lymph nodes); 1 case - bulky tumor mass in soft tissues of supraclavicular, subclavian, axillary, scapular areas and regional lymph nodes; 1 case - shin bone and soft tissues of this area; 1 case - neck and supraclavicular lymph nodes; 1 case - bulky tumor mass in mediastinum, lesions of pericardium, pleura, lung, mediastinal lymph nodes; 1 case - small bowel (with features of its acute obstruction). Treatment of MS consisted of the regimen for AML: induction therapy "3+7+7"(Idarubicin 12 mg/m^2 d 1-3, Ara-C 200 mg/m^2 d 1-7, Etoposide 75 mg/m^2 d 1-7), 2 cycles of consolidation therapy "HAI"(Idarubicin 12 mg/m^2 d 2, 4 plus Ara-C 3 g/m^2 every 12 hours d 1, 3, 5), 4 cycles of post-consolidation therapy "1+5+5"(Idarubicin 12 mg/m^2 d 1, Ara-C 200 mg/m^2 d 1-5, Etoposide 75 mg/m^2 d 1-5). All pts received intrathecal Mtx + Ara-C + Dexa. In 1 case of small bowel acute obstruction the first step of treatment was surgical. 2 pts received radiotherapy on tumor lesions areas. 1 patient received high-intensity chemotherapy and autologous stem cell transplantation after 2 cycles of consolidation therapy "HAI". Follow-up period was 16 months. Median OS and median RFS were not reached. 1 case of relapse was registered. RFS was 67% at 3 year_s follow-up period. Remission duration ranged from 1+ to 39+ months, life duration ranged from 4+ to 52+ months, all pts are alive at the time of analysis. *Conclusion*. MSs are characterized by various clinical features. Intensive chemotherapy for AML makes it possible to obtain encouraging results in treatment of MSs.

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CORRELATING OUTCOMES AND LONG-TERM SURVIVAL FOLLOWING CHEMOTHERAPY IN NORMAL KARYOTYPE AML WITH MOLECULAR RISK - SINGLE CENTRE EXPERIENCE

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Background. Patients with normal karyotype AML (NK AML) are a heterogeneous group and assessment for molecular genetic aberrations are important for prognostic differentiation. Presence of mutation involving internal tandem duplication of FLT3 gene (FLT3-ITDmut) is reported in 20-30% NK AML and is associated with poor outlook. In contrast, prognosis is considered good for patients who are FLT3-ITDmut negative but have mutations of NPM1 (NPMmut) or CEBPA (CEBPAmut) genes. *Aims.* We analyse the molecular profile in relation to therapeutic outcomes following chemotherapy in 35patients with NK AML treated between 2003-2007. *Methods.* Only patients less than 65 years old who received induction chemotherapy were included in study. Molecular analyses for mutations of FLT3-ITD, NPM1 and CEBPA were performed on bone marrow samples obtained at diagnosis. Standard induction therapy was idarubicin and cytarabine (IA3+7). Bone marrow assessments were performed on day 14 of induction and also upon recovery of neutrophil and platelet counts. Patients who failed to go into complete remission (CR) with 1 course of induction undergo a second cycle of IA3+7. Subsequent to achieving CR, further chemotherapy was administered with: 1 cycle of IA2+7 and 1 cycle of high dose cytarabine (HDAC). Following this, patients with persistent leukemia or who failed to go into CR after 1 cycle of induction chemotherapy or are FLT3-ITDmut positive undergo allogeneic stem cell transplant (alloSCT) if they have available sibling or match unrelated donors. All others undergo either autologous stem cell transplant (autoSCT), or 2 more cycles of HDAC. *Results.* The median age was 40years (15-63 years) and median follow up 30months (4 to 79months). Of 35 patients, overall rate of CR after 1 cycle of induction chemotherapy (CR1) was 26/35 (74%). Nine patients (26%) required 2 cycles of induction chemotherapy following which 8 achieved remission but 1 remained refractory and died of sepsis. Responses to chemotherapy and outcomes were correlated with presence or absence of the 3 molecular genetic aberrations as indicated in Table 1. Conclusion. In absence of FLT3-ITDmut, NPMmut or CEBPAmut positivity predicts for good responses to induction chemotherapy. Long-term outcomes are excellent following consolidation chemotherapy in this group as 80% remain disease free at 5 years compared with 5-year DFS of 14% for FLT3-ITDmut positive or triple negative patients (P=0.02). AutoSCT for consolidation is a reasonable approach for FLT3-ITDmut negative but NPM1mut or CEBPAmut positive patients.

Table 1.

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ACUTE MYELOID LEUKEMIA IN PATIENTS AGED 70 OR OLDER. EXPERIENCE AT A SINGLE CENTRE

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Background. The management of old patients with acute myeloid leukemia remains controversial, specially in those cases affecting very old patients (aged ≥70) in which the dilemma therapeutic abstention *vs.* treatment (with low or high intensity ones) is a major subject. *Aims.*- We present the experience in our centre with this group of patients in the period 1990-2009. *Methods.* During the period of study 85 cases were

diagnosed (relapses and FAB M3 cases were excluded). Patients were divided into 3 groups according to the treatment: supportive treatment, low intensity treatment (low doses Ara-C: 10 mg/m^2/12h s.c. days 1-21) and high intensity treatment (adapted ICE: Idarubicin 10 mg/m^2 days 1 and 3; Ara-C 100 mg/m^2/12h days 1-3; Etoposide 100 mg/m^2 days 1-3). *Results.* The mean age of patients was 76,6 years (70-97); sex distribution was 42 males and 43 females; mean Karnofsky index was 71,9 (30-100); 51 patients received treatment and 34 did not; overall survival was 6,9 months (median 2; 0,03-90+), significant differences were observed in the mean overall survival between the treated and no-treated groups (10 *vs.* 2,2 months respectively; P=0.01). In the low intensity group (31 patients) an overall response of 32,2% (6 CR, 4 PR, 14 NR and 7 not evaluable) was observed while in the high intensity one (20 patients) this overall response was 55% (9 CR, 2 PR, 4 NR and 5 not evaluable); no statistical differences were observed between both groups considering all subgroups of response (P=0,17), however grouping PR+CR *vs.* NR+not evaluable these differences were significant (P<0.01). Considering overall survival, no statistical differences were observed between the low and high intensity groups 7,1 (0,25-90+) *vs.* 14,4 (0,25-76+) months (P=0,15) respectively. *Conclusions.* Overall survival in the treated group is higher than in the non-treated one, differences reached statistical significance (P=0,01). Comparing both arms of treatment statistical differences were observed in the quality of response (PR+CR *vs.* NR+not evaluable), a higher proportion of complete responses were observed in the high intensity group (45% *vs.* 19,3%, respectively), however, if this circumstance will contribute to a longer survival is still unknown. Though no statistical differences have been observed in the overall survival between both groups of treatment, these differences are increasing along time.

1254

ABSOLUTE LYMPHOCYTE COUNT (ALC) IS A POWERFUL PREDICTOR OF OUTCOME IN PATIENTS WITH ACUTE MYELOID LEUKAEMIA (AML) RECEIVING INTENSIVE CHEMOTHERAPY

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Recent evidence has highlighted the value of the absolute lymphocyte count (ALC) as a useful prognostic indicator of outcome in patients undergoing intensive chemotherapy for treatment of haematological malignancy, notably acute leukaemia. We sought to study the relationship between the ALC and relapse-related mortality in 32 patients who had received intensive chemotherapy for a variety of haematological malignancies from 2005 to 2008 at the Royal Sussex County Hospital in Brighton. In particular, we tested the hypothesis that the ALC is a predictor of survival in acute myeloid leukaemia (AML) which comprised the major disease subgroup. We undertook a retrospective analysis of all level 2 patients treated with intensive chemotherapy at the Royal Sussex County Hospital. The period of study covered three years and included 32 patients, of whom 17 had AML. The ALC was available for all these patients at days 1, 8, 15, 22, 29 following the initiation of induction chemotherapy. We divided the patients with AML into two groups according to whether or not they were still alive at the time of the analysis. The survivors (9/17) had a mean ALC at day 29 of 1020/μL whereas the non-survivors had a mean ALC at day 29 of 430/μL. This was statistically significant using a Student's unpaired t-test (P=0.0067). The mean ALC of all 17 patients was 720/μL with the median value of 800/μL. All 9 survivors had an ALC at day 29 greater than the median of the non-survivors (400/μL). Conversely, all but one of the non-survivors had an ALC at day 29 less than the median of the survivors (900/μL). We also performed a retrospective analysis of 39 patients diagnosed with multiple myeloma to see if the ALC was predictive of outcome in this condition. Our data demonstrates that the ALC is not predictive of outcome in multiple myeloma. We conclude that the day 29 ALC is a simple and statistically powerful measurement of outcome for patients receiving intensive chemotherapy for treatment for AML that can serve as an independent prognostic indicator for improving risk stratification and management decision making in AML. We believe that the ALC reflects the underlying immune status of the patient and are currently investigating the mechanism by which this improves survival.

1255

RELAPSE OR REFRACTORY ACUTE MYELOID LEUKEMIA TRETED WITH AZACITIDINE

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Background. Relapsed or refractory acute myeloid leukemia (AML) has a poor overall prognosis, namely in older patientes. Patients with intermediate or poor risk cytogenetics who have relapsed more than once generally have a median survival of less than a year. A number of salvage chemotherapy regimens have been employed in these settings. The hypomethylating agent, azacitidine are generally well tolerated and have been demonstrated to produce responses in a small percentage of patients with relapsed or refractory AML. *Aims.* To investigate the efficacy and toxicity of azacitine in seven patients with relapse or refractory AML *Methods.* From Oct-08 to Dic-2009, 5 patients with refractory or relapse AML, were included. High-risk cytogenetics were found in, Hematological response was assessed according to the International Working Group Criteria for AML. *Results.* At baseline the median age was 67(21-76) years. Male/female ratio 1/4, an ECOG performance status of 0-1 (4 patients; 80%). The most frequent initial dose of AZA applied was 75 mg/m^2 (in whom the dosage schemes applied were: days 1-5 and 8-9 (4 patients) and days 1-5 (1 patients)in every 28 days cycle. AZA were administrated mostly subcutaneously (4 patients). The mean number of cycles administered was X (range 1-15). The overall treatment response was % (IWG 2006 criteria): 40 % partial response and 40 % hematological response, . Nevertheless, 40% achieved a stable disease. AZA was generally well tolerated. Grade 3/grade 4 adverse events documented in these patients, independently of their relationship to the active treatment, were febrile neutropenia (40%), rash (0%), vomiting or nauseas (20%), using antiemetic profilaxis. All patients treated with azacitidine presented burden cytopenias so hematologic adverse events couldn't be analized. Subsequent therapy with allogeneic HSCT in one of these individuals. *Conclusions.* Our results demonstrate that in the community-based setting, patients with relapse or refractory AML response to the treatment with azacitidine. We expect this evidence could offer in the near future evidences for a better prognosis for these patients in our environment prolonging their survival time and offering a better quality of life. Azacitidine is well tolerated in patients with AML and can often be given in an outpatient setting This therapy facilitated allogeneic HSCT as further therapy in responding patients.

Table.

Edad/sexo	Citogenética	Regímenes previos	Recaída/refractario
68/Female	-7	LMA>65; FLAG-IDA	Refractaria
76/Female	-7	CETLAM>70 (3 ciclos)	Refractaria
67/Female	Del 5, +8	CETLAM>70 (completo)	Refractaria
63/Female	Normal	FLAG-IDA+Mylotag 1 ciclo	Recaida refractaria
21/Male	-7	CETLAM<70(induccion); FLAG-IDA	Refractario

1256

CLINICAL AND BIOLOGICAL CHARACTERISTICS OF ADULT BIPHENOTIPIC ACUTE LEUKEMIA (BAL): A SERIE OF 45 ALGERIAN PATIENTS

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Background. BAL display features of both myeloid and lymphoid lineage. It is rare, actually well diagnose thanks to the immunophenotype. We present here the clinical and the biological characteristics and the out come of BAL in our experience. *Design and Methods.* We analysed 45 adult BAL patients (pts),diagnosed between January 2002 and December 2009 according to the European group for the Immunological characterization of leukemia (EGIL), among 429 adult acute leukemia pts diagnosed according to FAB classification. *Results.* During a period of 7 years, of 452 acute leukemia pts, 45 cases of BAL were diagnosed in our unit (10.4%), among them 33 (73.7%) were B lymphoid and myeloid (B/M), and 12 (26,6%) were T lymphoid and myeloid (T/M), no case of B/T or trilineage differenciation. The median age is 27 years old (15-81), the sex ratio is 1,6 (28 M/17 F). The median count of white cells, hemoglobin and platelet is respectively 30, 9 (1,1-239)×10^9/L, 81 (44-159)/L and

64 (9-477)/L, respectively. The median prevalence of blast in bone marrow was 94% (27-100). Nine patients (20%) have extra medullary infiltration, which most commonly affect the mediastinum, the central nervous system was affected in 4pts(8,8%).Lymph nodes was affected in 18pts (40%), spleen in 13pts (28,8%) and both in 11pts(24.4%). The incidence of CD34 antigen expression were 73,3%; 58.3% of them were T/M. Above Fifty patients who has a genetic profil, 3 has t (9-22), 2pts has t(8-21), and 1 a SIL- TAL transcript. The induction regimen was Vincristin, Daunorubicin and L Asparaginase in 42 pts (97,6%) with negative myeloperoxydase coloration, and Daunorubicine - Cytarabine in 3 pts (6,9%) with positive ones, 2 pts do not have any treatment. The complete remission rate was 81,5%.The median follow up is 5 years, twenty(44,4%) patients are still alive, 2 are in relapse, 25pts died (55.5%), 2 before treatement, 7 pts after relapse (16.2%), 7 are refractory (16,2%), 4pts (9.3%) in complete remission and 5 pts (11%) during the induction. The overall survival is 28,8%, The disease free survival is 26,6%. *Conclusion.* The prognostic of BAL pts is poor. They showed a most higher extra medullary infiltration, relapse and resistance to therapy after relapse. In our series the rate of death in complete remission is still too high, because of the remoteness of sanitary structure inducing late treatment of some infectious problems.

1257

BLASTIC PLASMACYTOID DENDRITIC CELL NEOPLASM (BPDCN): REPORT OF A CASE ASSOCIATED WITH CHRONIC MYELOMONOCYTIC LEUKEMIA

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Background. Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is a rare, aggressive systemic neoplasm originated from plasmacytoid dendritic cells (PDC), a hematopoietic-derived cell implicated in the regulation of innate and adaptive immunity. *Aims.* We describe the clinical and biological characteristics of a case of BPDCN associated with myelomonocytic leukemia. *Methods.* A 73 years old woman presented with cervical lymphadenopathy and nodular erythematous cutaneous lesions, associated with mild anemia (Hb 11.6 g/dL) and thrombocytopenia (120000/mm^3), white blood cell (WBC) count and LDH were normal. On histology, skin, lymph nodes and bone marrow were infiltrated by blastic PDC as defined according to their phenotype ($CD4^+CD56^+CD123^+BDCA2^+TCL1^+CD2AP^+BCL11a^+$, with coexpression of CD2 and CD7, but negativity for B-, T- and myelo-monocytic markers). Diagnosis of BPDCN was made. Cytofluorometric peripheral blood (PB) assay showed 8% of cells expressing CD2, CD4, CD7, CD56, and HLA-DR. Because of rapid progression with fever, organomegaly, disseminated cutaneous lesions and pleural effusion, MICE protocol (mitoxantrone, etoposide and cytarabine) was started. *Results.* Aplastic phase was complicated by Pseudomonas Aeruginosa sepsis and pneumonia resistant to antibiotic therapy; the patient remained febrile despite of the association of antimycotic drugs, with progressively increasing interstitial-alveolar infiltrations and pulmonary insufficiency. During the haematologic recovery she developed significant PB monocytosis (21.496/ mm^3) with 25% of atypical BM monocytoid precursors and 3% of BM blasts. PB and BM cytofluorometry and histology showed cells of monocytic lineage expressing $CD4^+CD56^+$ $CD11c^+CD14^+$, as well as CD123 and CD2AP, but negative for other BPDCN markers. BM dysplastic changes were also obvious. After steroid therapy the clinical conditions improved rapidly and monocytosis resolved, but one month later central nervous system relapse with marked liquoral blastic cell count occurred and patient died. *Conclusion.* In about 15%-20% of cases BPDCN has been reported to be associated with or develops into a myelomonocytic leukemia or acute myeloid leukemia; this study shows that partial sharing of markers between the two neoplastic populations may occur, thus suggesting that the two diseases are more than coincidental and may have a common cellular origin.

1258

ACUTE PROMYELOCYTIC LEUKAEMIA. CLINICO-PATHOLOGICAL PROFILE: EXPERIENCE OF AL-AMAL ONCOLOGY-HAEMATOLOGY CENTER IN QATAR

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Introduction. Acute promyelocytic leukemia (APL) is a subtype of Acute Myeloid Leukemia (AML). It has a characteristics clinical, morphological ,Cytogenetics and molecular pattern. Previously considered as the most devastating subtype of AML now the most treatable of all subtypes. No data has been published about APL in Qatar.

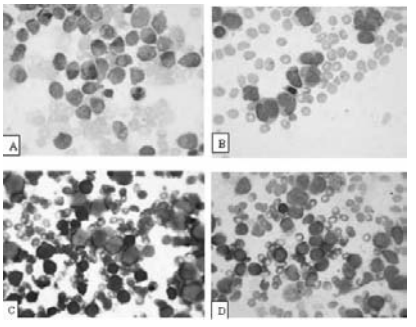

Figure. Classical hypergranular APL (A) microgranular APL variant (B), hyperbasophilic APL variant (C), M3r APL variant (D).

Patients and Methods. This is a retrospective study describe the experience of Al Amal hospital (the adult Oncology/Hematology center in Qatar) with APL patient seen during the period from January 2006 to May 2008 .The diagnosis was established by combined morphological examination, immunophenotyping & cytogenetic studies. *Results.* Out of thirty four AML cases diagnosed, eleven were classified as APL reaching a frequency of (32.2%). DIC was common at presentation (91%). Severe thrombocytopenia seen in 72.7%,leukocytosis in 54.5% and severe anaemia in 45.4%. Only two patients were of the classical hypergranular type. In the remaining nine patients(81.8%) three morphological subtypes were recognized: Microgranular variant in six patients, Hyperbasophilic in two patients and the variant with regular nuclear outline (M3r) in one patient. Translocation (15;17) was detected in 62.5% of the cases analyzed. *Conclusion.* This study shows that APL constitutes a significant proportion of AML in Qatar with significant morphological heterogeneity and predominance of variants APL having unfavorable presenting features.

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EVALUATION OF PLASMA LEVEL COPPER,ZINC,CU/ZN,VEGF IN PATIENT WITH AML BEFOR AND AFTER TREATMENT

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Background. Copper and Zinc are the elements that have numerous physiologic activities. Copper has an important role in angiogenesis. The effect of Copper is applied by increasing of VEGF. Serum level of copper increases cancer incidence, progression and recurrence of Hodgkin's lymphoma, sarcoma, leukemia and lung, liver, breast tumors. *Aims.* The aim of this study was measuring blood levels of copper and zinc and the ratio of Cu/Zn, as well as VEGF levels before and after treatment in patients with acute myeloid leukemia. *Methods.* 30 AML newly diagnosed patients in Shahid Ghazi Tabatabai enrolled in this clinical trial. On the first day blood sampled for copper, zinc and VEGF assay and flow cytometry. Treatment protocol was (7×3) ARA-C 100 mg/m^2 for 7 days and Cerubidin 45 mg/m^2 for 3 days. In complete remission blood samples were collected for evaluation of copper, zinc and VEGF and were sent to Biochemistry Laboratory in school of medicine for analysis. *Results.* Of total 30 AML patients 14 (7 / 46%) were female, and 16 (3/53%) were male. patients ranged in aged from 16 to 53 years, with a median age of 9.1±9.35 years. The mean serum level of copper and zinc, before and after treatment showed significant difference ($P<0.05$) ,as well as mean ratio Cu/Zn .There was significant difference between the Mean VEGF level before and after treatment ($P<0.05$). *Conclusions.* In our knowledge based on searching data bases there is not any report indicating copper and zinc serum level correlation before and after treatment in AML patients, and this may be the unique one in this era. We didn't find relationship between copper, zinc serum level, their ratio and VEGF in AML. We expected the increased serum copper noticed by increasing VEGF level that indicates the positive effects of copper in the incidence of malignancies.

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BLASTIC PLASMACYTOID DENDRITIC CELL NEOPLASM - DIAGNOSTIC CASE REPORT

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Blastic plasmacytoid dendritic cell neoplasm (BPDC) formerly known as $CD4^+CD56^+$ hematodermic tumor is a rare hematological neoplasm derived from the precursors of plasmacytoid dendritic cells. A 35 year male patient was examined at our department for unexplained pancytopenia. He had a 3 months history of fatigue and febrile episodes, which required antimicrobial therapy. At the same time the patient noticed a painless 3 cm induration on his right thigh. Blood count investigation revealed leucopenia, thrombocytopenia and mild normocytic anemia: WBC 1.68×10^9/L, Neut 0.53×10^9/L, RBC 3.81×10^{12}/L, HGB 116 g/L, PLT 103×10^9/L. Cytological examination of peripheral blood and bone marrow raised the suspicion of acute myeloid leukemia without maturation (4%, respectively 86% myeloperoxidase negative blast cells). Flow cytometry immunophenotyping of bone marrow aspirate and histological examination of skin biopsy however set the diagnosis of blastic plasmacytoid dendritic cell neoplasm. HAM induction therapy achieved complete hematological remission without complete blood count recovery, followed by I. HAM consolidation and allogeneic hematopoietic stem cell transplantation from unrelated donor. Actually the patient is in hematological remission with detectable minimal residual disease. *Conclusion.* Morphological and cytochemical examination but also extensive immunophenotypic and histological/immunohistochemical analysis is mandatory for definitive diagnosis of BPDC.

1261

LIPOSOMAL CYTARABINE FOR NONLYMPHOBLASTIC MENINGITIS - ROMANIAN WORKING GROUP FOR ADULT ACUTE LEUKEMIA EXPERIENCE

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Background. Liposomal cytarabine is an attractive drug for CNS leukemia treatment, given the ability to reduce the frequency and total number of IT treatments. The efficacy of liposomal cytarabine in lymphomatous meningitis prophylaxis and treatment was reported. Central nervous system involvement in acute myeloblastic leukemia is rare and the treatment of meningeal disease remains a challenge. In addition to the standard approach, liposomal cytarabine (Depocyte) permits to decrease the frequency of lumbar punctures, without loss of efficacy, because the intrathecal levels of the drug remain cytotoxic for up to 14 days. *Material and Methods.* We present 3 patients with acute myeloblastic leukemia (M2, M4 and M5 FAB) and SNC leukemia. There were 2 males and 1 female, aged 42, 52 and 65 respectively. The range number of IT administrations was 5 at 14-21 days well tolerated. *Results.* All patients achieve complete liquor blasts clearance after first Depocyte administration. The female, aged 65 years dead with hematological relapse at 12 months after diagnosis. The male 42 year old received related stem cell transplant and is alive in complete remission at 10 months, without CNS leukemia. The third patient is alive at 5 months with high doses systemic chemotherapy without SNC relapse, on the waiting list for unrelated stem cell transplant. *Summary*. Myeloblastic meningitis would be a new indication for liposomal cytarabine 50 mg given IT every 14 days.

1262

DEVELOPMENT OF MULTI-COLOR INTRACELLULAR PANELS IN CHRONIC LYMPHOID LEUKEMIA (CLL) USING ZAP-70 QUANTUM DOT-CONJUGATED ANTIBODIES

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Background. B-cell chronic lymphocytic leukemia (CLL) has a highly variable clinical course. Identified clinically useful prognostic markers include IgVH mutational status, and Zap-70 and CD38 expression. Recent studies have shown that Zap-70 expression may act as a surrogate for IgVH mutational status in CLL. Combined analysis of Zap-70 and CD38 has provided more refined prognostic information. Flow cytometry presents advantages over other methods to detect Zap-70. *Aims.* The goal of this study was to develop and optimize a new methodological approach for intracellular staining with antibodies against Zap-70 conjugated with Quantum dots (QDs) nanoparticles suitable for development of multi-color panels. *Methods.* We analyzed the Zap-70 expression levels in 38 fresh and cryopreserved samples from two groups of patients with newly diagnosed or treated CLL from two outpatient clinics (MD Anderson Cancer Center, Houston, USA and Volgograd Clinical Oncology Center, Volgograd, Russia). The median age of the patients was 65.4 years (range 48÷84). The cohort of CLL patients included 16 untreated and 22 patients on different therapy treatments. The expression of Zap-70 in B-CLL cells was analyzed by flow cytometry using Zap-70 antibodies (clone 1E7.2) conjugated either with QD655 or organic fluorophores (fluorescein) in 3-5 colors combinations with CD19, CD5, CD3, CD38 monoclonal antibodies. % of Zap-70 positive B-cells was calculated using T-lymphocytes from CLL patients as positive controls. Samples were acquired with FACSCanto flow cytometer (BD Bioscience, San Jose, USA). The IgVH mutation status was determined in 23 patients. Shortly, high molecular weight DNA was isolated by phenol-chloroform method. Amplification of VDJH rearrangements was performed, and PCR products were purified and directly sequenced. Germline VH, DH and JH segments from VDJH rearrangements were identified by comparison with database and sequences containing more than 2% deviation from the germline sequence were considered as somatically mutated. Spearman rank correlation was used to analyze correlations and value of $P\leq0.05$ was considered significant for all statistical calculations. *Results.* Out of seven tested commercially available fixation/permeabilization buffers only two present an appropriate combination for the QDs delivery across cell membranes. The percentage of Zap-70 positive B-cells as determined by the QD655-conjugated Zap-70 antibody strongly correlated with the Zap-70 expression levels determined by standard method (Zap-70 conjugated with organic fluorophore)($r=0.593$, $P\leq0.05$) at fresh peripheral blood samples from CLL patients. Combined analysis of QD655-conjugated Zap-70 and IgVH mutational status at CLL patients yielded discordant results in 27.2% cases (vs 33.3 for FITC-Zap-70 antibody panel), whereas 36.3% were concordantly positive and 27.2% concordantly negative. Mean fluorescence intensity (MFI) did not diminish with the increase of fluorophores number in the panel, when QD655-conjugated antibodies were used to detect Zap-70. Positive to negative signal ratio is higher when intracellular QDs nanocrystals were used in comparison to standard fluorophore combination. *Conclusions.* There is a first successful Zap-70 protocol using QD-conjugates for intracellular staining. The QDots conjugated antibodies are advantageous in multiplexing comparing with organic fluorophores, and can be used for evaluation of combination of prognostic markers for leukemia.

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AN *IN VITRO* TEST PREDICTIVE OF THE IN VIVO B CELL RITUXIMAB-TRIGGERED DEPLETION, UNDERLINES THE POTENTIAL THERAPEUTIC INTEREST OF A RECOMBINANT ANTI-CD20 IGA IN B-CLL PATIENTS

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Background. Most of therapeutic monoclonal antibodies (thmAb) are built with an IgG1 constant part, in order to trigger complement activation, antibody-dependent cell cytotoxicity (ADCC), and allow long half-life through the interaction with the Neonatal Fc receptor of Ig. One of them, rituximab, targeting the CD20, is widely use to treat lymphomas, auto-immune diseases and B chronic lymphocytic leukaemia (B-CLL). In the latter indication, the rituximab-induced B cell depletion varies greatly from one patient to another. *Aims.* With the aim to predict the *in vivo* efficacy of rituximab in B-CLL patients, we adjusted an *in vitro* test based on the adding of rituximab to whole blood. By means of this whole blood assay, we evaluated the *in vitro* B cell depletion induced by a recombinant anti-CD20 IgA antibody built with variable parts of rituximab, which have been evoked as an alternative isotype. *Methods.* The rituximab or its IgA recombinant homologue, were added to whole blood samples of B-CLL patients (N=50). After three hours of incubation in presence or absence of anti-CD20 antibodies, flow cytometry absolute counts of CD19+CD3- cells allowed to calculate the B-CLL cell depletion. For six patients, we compared the *in vitro* and *in vivo* B cell depletion. *Results.* For six patients, the *in vitro* rituximab induced B-cell depletion revealed by the whole blood assay paralleled the *in vivo* therapeutic effect. The *in vitro* rituximab induced B cell depletion revealed by the whole blood tests with B-CLL blood samples (N=50) did not correlate with the one induced by its IgA counterpart. The *in vitro* B cell depletion induced by the anti-CD20 IgA was either stronger than, weaker than, or equivalent to the RTX-induced depletion for 8, 13 and 29 patients, respectively. *Conclusions.* We demonstrate that the IgA recombinant anti-CD20 antibody is efficient to deplete B-CLL cells *in vitro*. In some cases, the IgA appeared more efficient than the rituximab. Because the *in vitro* test results paralleled the *in vivo* RTX induced B-CLL cells depletion, one can suppose that some B-CLL patients could benefit more from a treatment with the recombinant IgA anti-CD20 than from rituximab.

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MONITORING OF NATURAL KILLER (NK) CELLS PHENOTYPE AND ANTITUMORAL ACTIVITY IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. Chronic lymphocytic leukaemia (CLL) is one of the most common leukaemias in adults in the developed world. Despite advances in anticancers therapeutics, CLL remains incurable. The ability of NK cells to control different haematological malignancies has been demonstrated *in vitro* and *in vivo*. However, alterations of these effector cells have been described in patients with haematological malignancies that might explain an escape from innate immune surveillance.Recently, our group has reported that patients with acute myeloid leukaemia defective interactions receptor-ligand in NK cells possibly due to a defective expression of activating receptors (NKp30 and NKp46) that could represent an immune escape mechanism for leukaemic cells. There is only limited information about the possible defiency of NK cells in CLL. *Aim.*The aim of this work is to evaluate the activating receptors expression in order to determine if it represents an escape mechanism of the immune response in CLL. *Methods.* We investigated the receptor repertoire and measured NK cell activity in peripheral blood of 40 untreated patients with CLL, 30 stage A and 10 stage B/C, and 17 healthy donors (age-matched). The expression of activating receptors (NKRa) that mediate NK cell recognition and lysis of leukemic cells (NKp30, NKp46, NKG2D and DNAM-1) was investigated by flow cytometry. The expression of inhibitory receptors ((NKRi), (KIRs, ILT2, NKG2A)) was also analysed. *Results.* The expression of activating receptor NKp30 was significantly decreased in the patients with stage A (P=0.0005) or with stage B/C CLL (P=0.0097) compared to healthy donors. In CLL NK cells, the expression of NKRi and other NKRa did not differ from healthy donors. However, the cytotoxic activity of NK cells was not significantly different in patients with stage A or stage B/C CLL compared to healthy donors. *Conclusion.* A decreased expression of a major activating receptor NKp30 was observed in CLL independently of the stage. However, this alteration does not affect the lytic activity of CLL NK. In CLL, therapies improving NK functions including monolonal antibody, proteasome inhibitor or immunomodulatory drugs (IMIDs) represents a novel strategy.

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TNFRSF4 (OX40) EXPRESSION IN CLL CELLS: EVIDENCES OF A PROGNOSTIC SIGNIFICANCE OF GENE AND PROTEIN OVEREXPRESSION

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Background. OX40 is an antiapoptotic molecule of activated T lymphocytes. There are not published data reporting OX40 in B-lymphocytes. The presence of high seric levels of OX40 in CLL was thought to be related to the expansion of activated T lymphocytes. However OX40 expression in CLL cells was not tested. *Aims.* to evaluate the expression of OX40 in CLL cells and its prognostic value. *Methods.* OX40 mRNA levels was evaluated by means of real time PCR in $CD19^+$ peripheral blood cells, isolated by magnetic cell separation (Mylteni Biotech, Auburn, CA), in 55 untreated CLL patients characterized for FISH, IGVH mutational status, ZAP70, CD38, need of therapy, and in 10 healthy controls. Ox40 protein expression was assessed by immunohistochemistry (IHC) on bone marrow biopsies performed at diagnosis in a different cohort of 101 CLL cases, in order to analyze retrospectively the relationship between protein expression and outcome. For IHC we employed the previously validated (www.proteinatlas.org) antibody TNFRSF4/OX40 Clone BER-Act35, Santa Cruz Biotechnology, CA, USA. Protein expression were defined according to the following scoring system: percentage of positive proliferation centers (<50% = score 0; > 50% = score1); percentage of positive cells into the proliferation centers (<50% = score 0; > 50% = score 1); positivity of neoplastic cells outside the proliferation centers (none = 0, focal = 1, diffuse = 2). Score 1-2 defined low expression; score 3-4 defined high expression. Patients evaluated for IHC had classic CLL, median age of 60 y (range 31-80); Binet stage A 72%; stage B 20%; stage C 8%. With a median follow-up of 468 days, (range 124-3860), 54/101 patients were treated and 11/101 died. *Results.* OX40 gene was overexpressed in CLL patients in comparison with normal individuals (Fc >2; P<0.01 T test). Between CLL cases having different features, OX40 gene was overexpressed in IGVH unmutated, ZAP 70^+ and $CD38^+$ cases, and in patients requiring therapy (false discovery rate <5%, calculated by means of Significant Analysis of Microarray). OX40 protein high expression relates to Binet Stage B and C (P=0,016). Patients having protein high expression, as compared with patients having low expression, had lower rates of 2 years time to treatment (46% *vs.* 81% P=0.001); and 5 years overall survival (80 and 96 % P=0.058). Summary/*Conclusions.* Over-expression of OX40 is identifiable in CLL cells. OX40 mRNA levels were higher in patients with unfavorable features. Since high levels of OX40 protein expression predicts for poor outcome, IHC for OX40 should be considered in prospectical studies for clinical application. Further investigations are needed to clarify if OX40 in B cells activates the same antiaptotic pathway than in T cells. In such a case OX40 might be considered as a potential target for therapy.

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REGULATORY T-CELL NUMBER IS CORRELATED TO FEATURES OF ACTIVE DISEASE IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

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Background. Naturally arising $CD4^+CD25^+$ regulatory T-cells (Treg)

actively maintain immunological self-tolerance. A reduction in the number or function of these cells can also elicit tumour immunity. Several studies evidenced that the immune system in patients with chronic lymphocytic leukemia (CLL) is deficient. *Aims.* In the current study we have evaluated, by means of a multiparametric flow cytometric approach, the circulating Treg cell number in 80 patients with previously untreated CLL and in 40 normal subjects. *Methods.* CD4+CD25+high density cells were gated and evaluated for CD127 expression at low or undetectable levels to analyze only Treg cells. *Results.* A lower percentage number of Treg cells was detected in CLL patients (mean number 0.43%, range 0.02-1.2%) than in controls (1.13%, range 0.2-2.1%). On the contrary, when evaluated as absolute number, CLL patients showed a higher number of Treg cells (mean number 70.8/μL, range 3-880/μL) compared to controls (mean number 23.2/μL, range 4.4-41/μL). A correlation of Treg cell absolute number was also found with more advanced Rai clinical stage (P<0.0001), absolute CD38 positive B-cell number (p 0.02), and more elevated LDH levels (p 0.037). No correlation, however, were found with ZAP-70 expression, IgVH mutational status and cytogenetic abnormalities. Only in 2 patients Treg cells were found at very low levels. These patients suffered from a concomitant autoimmune disorder (autoimmune haemolytic anaemia and idiopathic thrombocytopenic purpura) at the moment of analysis. *Conclusions.* Our data showed that Treg cells are higher in CLL patients and correlate with disease status rather than prognostic factors themselves. This subset of T-cells is probably involved in the crucial mechanism of pathogenesis and progression of CLL and a therapeutic intervention targeting these cells need to be explored.

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HUMORAL IMMUNE RESPONSES AGAINST THE ROR1-PROTEIN SHOW PROGNOSTIC SIGNIFICANCE IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. The receptor tyrosine kinase-like orphan receptor 1 (ROR1) protein is a newly characterized oncofetal antigen in patients with chronic lymphocytic leukemia (CLL). Since a significant proportion of CLL patients never require antitumor therapy, we hypothesized that an autologous tumor-specific immune response is capable of controlling malignant disease. *Aims.* We investigated whether humoral immunity specific for the CLL-associated antigen ROR1 has a prognostic value in CLL. *Methods/Results.* Among sera of 87 untreated patients with CLL, 41 (47.1%) had detectable ROR1-antibodies which were reactive for at least one specific ROR1 epitope. Patients with humoral responses compared to patients with non-reactive sera had a longer progression-free survival (PFS)(P<.001). IgG subclass analyses showed a predominant IgG1 and IgG3 response. ROR1-antibodies were capable of recognizing and selectively killing ROR1-expressing CLL cells in complement-mediated and antibody-dependent cellular cytotoxicity (ADCC) assays. In the analysis of 44 CLL patients after allogeneic hematopoetic stem cell transplantation, twenty-nine showed significant titers of anti-ROR1 antibodies which specifically recognized the extracellular domain of the protein; suggestive for a potential role of anti-ROR1-directed immune responses to the graft-versus-leukemia effect in CLL. *Summary/Conclusions.* Our data suggest that spontaneous tumor-specific humoral immune responses against ROR1 exist in a significant proportion of CLL patients and that superior PFS in those patients could reflect autologous immune control.

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CYCLIN D1 EXPRESSION IN B-CELL LYMPHOMAS: A COMPARATIVE STUDY

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Background. Cyclin D1, an important component of cell cycle machinery and a protein with known oncogenic potential, presents at extremely low level in mature B-lymphocytes. Its overexpression might be important for B-cell oncogenesis. Indeed it happens in mantle cell lymphoma as a result of translocation t(11;14)(q13;q32) and was reported in some cases of CLL. Growing body of evidence demonstrate the possibility that it might occur in other B-cell lymphomas, however, the direct measurement using PCR did not give definite *Results.* One of the reasons for this uncertainty might be insufficient sensitivity of RT-qPCR when using single reference gene. *Aims.* The aim was to determine variations in cyclin D1 expression level in different B-cell lymphomas and to select appropriate reference genes for cyclin D1 validation in lymphoid tissue by RT qPCR. *Methods.* cDNA samples from 100 patients with B -CLL, MCL, CD5-negative B -cell lymphomas were used in this study as well as cDNA from reactive hyperplasia specimens and sorted B cells of 4 healthy volunteers. Diagnoses were established by 3-color flow cytometry and immunohistochemistry. The samples were stained for CD5, CD19, CD23, FMC7, CD45, CD20, CD22, CD38 and κ-/λ- light Ig chains according to the routine protocol. Data on cyclin D1 expression were assessed with real-time quantitive PCR with SYBR Green I. cDNA quantity of cyclin D1 was normalized using the geometric mean of most stable referent genes as normalization factor, and recalculated for tumor cells population using flow cytometry data. *Results.* YWHAS, ubc and HPRT demonstrated high stability in the whole cohort of patients (σ<1.73) and were selected as the primary set of reference genes for evaluating expression level of cyclin D1 in lymphoid tissue. The relative normalized quantity of cyclin D1 cDNA in sorted normal B-cells varied from 0.00003 to 0.0005, normalized cDNA quantity in unsorted specimens ranged from 0.00008 to 0.0009. MCL samples demonstrated high expression level and significant variance of cyclin D1 (n=31, range 0.20 - 23.13, median 0.994). Besides, compared to the reactive lymphoid tissue (n=18, median 0.0003) significant elevation of cyclin D1 was observed in B-CLL (n=33, median 0.014), and even in CD5-negative lymphomas (n=16, median 0.003). Addition of two remaining reference genes (b-actin and GAPDH) to normalization factor led to 10-fold increase in variance among CD5-negative lymphomas' group, and impaired the discrimination between CD5-negative lymphomas and reactive specimens. To allow a closer comparison of our results to others, we recalculated our data using ΔΔCt method with YWHAS as a sole reference gene. The variance in levels of cyclin D1 in all groups increased, and the difference between CD5-negative malignancies and reactive specimens became less significant (P> =0.0004, paired Mann-Whitney test). *Conclusion.* Quantitative RT qPCR allows accurate evaluation of gene expression in malignant B lymphocytes and B cells of reactive hyperplasia when using several reference genes for data normalization. We for the first time demonstrate statistically significant elevation of cyclin D1 expression in a variety of B-cell lymphomas.

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CD74 EXPRESSION CORRELATES WITH ZAP70 PROTEIN EXPRESSION BUT NOT WITH CD38 EXPRESSION AND IGVH MUTATIONAL STATUS IN CHRONIC LYMPHOCYTIC LEUKAEMIA PATIENTS

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Background. Chronic lymphocytic leukemia (CLL) is a heterogenous disease with different clinical course. New prognostic markers are still needed to define subgroups of patients with poor prognosis. CD74, known as the invariant chain, is a non-polymorphic glycoprotein with many immunological functions: major histocompatibility complex (MHC) class II chaperone, a receptor for macrophage migration inhibitory factor (MIF) and acts as accessory-signaling molecule involved in malignant B-lymphocytes proliferation and survival. *Aim.* The aim of this study was to determinate expression of CD74 in untreated CLL patient comparing to control healthy group and comparison of expression to already known prognostic factors in CLL, such as expression of ZAP-70 protein, CD38 and immunoglobulin variable heavy chain (IGVH) mutational status. *Methods.* Blood samples from 91 untreated patients with CLL (44 women and 47 men), aged 44-88 year (median age 68) were analyzed comparing to 28 matched healthy controls (15 women and 13 men). The study was provided with dataset of biological and clinical variables of CLL patients. Using flow cytometry the expression of CD74, as well as ZAP70 protein and CD38 was assessed on PBMC isolated from blood taken from patients during routine blood tests. All the samples were also tested using direct sequencing following ERIC recommendations on IGHV gene mutational status analysis in chronic lymphocytic leukemia. Statistical analyses were performed using statistical package EPIINFO Ver. 3.4.3 (Anova, Kruskal-Wallis test). The 'p' values <0.05 were considered statistically significant. *Results.* We observed significantly higher expression of CD74 in chronic lymphocytic leukaemia patients than in control group (mean value 9.4% *vs.* 0.35%; median 7.48% *vs.* 0.24%; SD 9.18 *vs.* 0.312; P<0.00001). There was significant positive correlation between expression of CD74 and ZAP-70 protein (r=0.28, P=0.009). CD74 correlated also with albumins (r=-0.21, P=0.081) and CRP (r=0.38, P=0.001). The expression of CD74 did not correlate with clinical features such as: leukocytosis (r=0.17, P=0,121), hemoglobin level (r=-0.03, P=0.762), platelet level (r=-0.16, P=0.153), lymphocytosis (r=0.1, P=0.393),

bone marrow lymphocytes (r=0.28, P=0.121), LDH (r=-0.02, P=0.886), beta-2-microglobulin (r=0.12, P=0.278). No correlation was found between expression of CD74 and CD38 (r=-0.04, P=0.741). Among the patient with mutated and unmutated status of IGVH there were no differences in CD74 expression (mean 8.62% *vs.* 9.67%, median 7.52% *vs.* 6.86%, SD 8.92 *vs.* 9.54, P=0.608). Expression of CD74 did not influence the need for treatment introducing in CLL patients (r=0.07, P=0.658). *Conclusions.* In our study we showed significantly higher CD74 expression in CLL patients comparing to controls with positive correlation between CD74 and ZAP-70 protein expression. CD74 measurement can help in stratification of patients to risk groups.

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HS1 EXPRESSION DOES NOT CORRELATE WITH ZAP70 PROTEIN, CD38 EXPRESSION AND IGVH MUTATIONAL STATUS IN CHRONIC LYMPHOCYTIC LEUKAEMIA PATIENTS

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Background. Chronic lymphocytic leukemia (CLL) is the most common B-cell malignancy in adults and is characterized a heterogeneous clinical course. Although many prognostic markers have been already discovered still further research are conducted to select groups of patients with poorer outcome. Hematopoietic lineage cell-specific protein 1 (HS1). It has been shown to be functionally involved in proliferation and apoptosis of B- and T-cell receptor activation. Different expression of HS1 was observed in CLL patients. *Aim.* The aim of this study was to determinate expression of HS1 protein in untreated CLL-patient comparing to control healthy group and comparison of expression to already known prognostic factors in CLL, such as expression of ZAP-70 protein, CD38 and immunoglobulin variable heavy chain (IGVH) mutational status. *Methods.* Blood samples from 88 untreated patients with CLL (44 women and 44 men), aged 44-88 year (median age 68) were analyzed comparing to 28 matched healthy controls (15 women and 13 men). The study was provided with dataset of biological and clinical variables of CLL patients. We assessed HS1 expression using western-blot analysis. In flow cytometry the expression of ZAP70 protein and CD38 was determined on PBMC isolated from blood taken from patients during routine blood tests. All the samples were also tested using direct sequencing following ERIC recommendations on IGHV gene mutational status analysis in chronic lymphocytic leukaemia. Statistical analyses were performed using statistical package EPIINFO Ver. 3.4.3 (Anova, Kruskal-Wallis test). The 'p' values <0.05 were considered statistically significant and those between 0.05 and 0.1 as indicative of a trend. *Results.* 76 CLL patients expressed HS1 protein comparing to 2 patients from control group. We observed significantly higher expression of HS1 protein in chronic lymphocytic leukaemia patients than in control group (P<0.0000001). We observed strong correlation between HS1 expression and clinical parameters: WBC (r=0.27, P=0.0129), HGB (r=-0.22, P=0.0465), platelets (r=0.-23, P=0.0310), beta-2-microglobulin (r=0.24, P=0.00342). HS1 expression did not influence the need for treatment introducing in CLL patients (r=-0.01, P=0.548). There was no correlation between HS1 and CD38 and ZAP-70 protein expression, but positive trend was observed (r=0.19, P=0.0891 and P=0.0866 for CD38 and ZAP-70 protein respectively). *Conclusions.* In study we showed significantly higher expression of HS1 comparing to control group, but without significant correlation with known prognostic factors.

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EPIDEMIOLOGICAL AND GENETICAL ANALYSIS IN A SERIE OF B-CLL PATIENTS: EPIGEN STUDY

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Background. The B-CLL, is the most common type of leukaemia diagnosed in the West countries (30%), the incidence in Europe is: 0.9-2.4 cases/year/100,000 inhabitants. Immunophenotypic, genetic and molecular markers allow their inclusion in different prognostic groups. OBJECTIVES: To determine the incidence in a year of CLL cases in a geographical area of Northern Spain with 4,686,175 inhabitants. To analyze the demographic, clinical, immunophenotypic and molecular markers and to relate them with gene expression profiles obtained with an oligonucleotide microarray. MATERIAL AND *Methods.* A prospective, descriptive, and analytical and cross section of incidental cases of B-CLL in Aragon, Navarra, Basque Country and Cantabria during the period: 1/09/2007- 31/08/2008. The study involved 13 hospitals that have collected the clinical characteristics at diagnosis and biological samples for DNA, RNA and serum extraction. The study has the approval of the Committee on clinical trials and investigation of Aragon (CEICA) and patients have signed IC. We designed a database for the study where the variables are collected: demographic, staging (Rai / Binet), lymphocyte count, immunophenotyping (IP) (CD38, Zap70), genetic testing (CG) and molecular (Ig VH genes mutation), study of gene expression profiles by the Hematochip platform (539 genes), follow-up to progression, development of primary tumors and response to treatment. *Results.* We collected data from a total of 96 new patients. The preliminary analysis includes 61 cases: men (H) 31 (50.8%) females (M) 31 (50.8%). Average age: 70.8 years (41-91) Males: 63.3 years and Females: 71.6 years. 52.4% were older than 70 years. Staging at diagnosis (Binet and Rai): A0 68.8%, 13.1% AI, AII 3.2%, 1.6% BI, BII 8.1%, BIII 3.2%, 3.2% CIV. 13.1% starts with splenomegaly. 29.5% had no lymph node involvement or extranodal affectation. CG: del13q (18.0%), trisomy 12 (18.0%), del11p (6.5%), del17p (1.6%), normal (45.9%). IP: CD38 positive (22.9%), Zap70 positive (16.3%). Ig VH mutated (21.3%). In 87 samples gene expression profiles have been studied, and differentially expressed genes were observed with statistical significance between different groups: non-mutated IgVH *vs.* mutated, del13q *vs.* normal karyotype, trisomy 12 *vs.* normal karyotype, positive CD38 *vs.* negative and positive Zap70 *vs.* negative. Among these genes, CFL1 has been validated by RT-PCR and found over-expressed in the group of patients with non-mutated IgVH genes. *Conclusions.* 1) The demographic data are similar to the control series and previous studies in greater sample size. We did not find differences by gender. Females were older than males. 2) Diagnosis in early stages (A / 0, A / I). 3) The more common genetic aberrations were: del13q and trisomy 12. Normal kariotype apparently is related to early stages, absence of spleen enlargement and B symptoms, typical morphology and predominance of IgVH gene mutation and CD38 and Zap70 negatives. 4) The analysis of gene expression by Hematochip platform showed differences between the analyzed groups. It is essential to go on studying the prognostic significance of identified genes.

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IMMUNOPHENOTYPIC PATTERNS IN CHRONIC LYMPHOCYTIC LEUKEMIA (CLL): RELATIONSHIP WITH GENETIC FEATURES AND MUTATIONAL STATUS

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Background. CLL has an heterogeneous clinical course. Well-established prognostic factors include genetic, molecular and phenotypic markers. The mutational status of IGVH and use of specific VH segments are important predictors of behavior. Flow cytometry (FC) is the most practical tool for prognostic stratification, including ZAP70 and CD38; recently it has been suggested that different combinations of other phenotypic markers may distinguish between stable and aggressive disease. *Aim.* To investigate a possible association between non typical phenotypes and other prognostic markers we characterized a series of CLL patients using FC, FISH and IGVH sequencing. *Methods.* 123 consecutive patients (47 females, 76 males, median age 66, 36-89 yo), diagnosed according to the 2008 NCI-WG criteria and from whom phenotypic and molecular data were available, were analyzed. FC was performed in 92 peripheral blood (PB) and 31 bone marrow (BM) samples, using various combinations of the following moAb: CD19, CD5, CD20, FMC7, CD79b, CD22 and IgLambda/Kappa. A typical phenotype was defined as strong CD5 and CD23 expression, with a dim surface Ig, CD20 and CD22 and weak or negative CD79b and FMC7. Patients were considered to have atypical phenotypes when the expression of ≥2 of these markers was aberrant. FISH for t(11;14) (LSI IGH/CCND1), performed in 20/25 atypical CLL cases, was negative. FISH for del13q14 (LSI

D13S319), tris12 (CEP12), del11q22 (LSI ATM) and del17p13 (LSI p53) was performed in 93% cases. DNA was extracted from mononuclear cells or whole blood. PCR amplification of IGVH rearrangements was performed with FR1 primers, using the BIOMED-2 strategy. Sequence analysis was done using IMGT data-base, following ERIC group recommendations. *Results.* 25/123 (20%) patients had an atypical phenotype, with a median number of 4 (2-7) aberrations. The most frequent were strong expression of CD22 and CD20 (92%,), strong monoclonal Ig expression (84%), strong CD79b expression (76%) and absence of CD23 (60%). Only 4/25 (16%) cases were heterogeneous/negative for CD5. CD38 expression differed between typical (42%) and atypical (9%) cases (P=0.005). 20/25 atypical cases (80%) were mutated, as compared to 59/98 (60%) typical ones. The incidence of somatic hypermutation was similar between the two groups (P>0,05, Fisher exact test). We further analyzed VH segment usage in 97 pts and found that in typical CLL VH1-69 predominated (16%, 15/97), followed by VH3-30 and VH3-7 (9%, 9/97 cases for each). Interestingly VH1-69, which is known to have an increased frequency among non-mutated cases, was absent from the atypical group. The incidence of cytogenetic abnormalities (46% and 30% for del13q14, 16 % and 35% for tris12, 12% and 4% for del11q22 and 6% and 4% for del17p13 in typical and atypical cases, respectively) was similar between the 2 groups (P>0.05 in all cases). *Summary/Conclusions.* CLL with a typical phenotype do not differ from those with a lymphoma-like pattern in terms of cytogenetic alterations and IGVH mutational status. Interestingly, CD38 expression and VH segment usage may be distinct in these 2 groups. Further studies are needed to confirm these findings and access their significance.

J Caetano, M Sebastião with equal contribution.

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ASSESSMENT OF APOPTOSIS CAUSED BY RESVERATROL IN COMBINATION WITH PURINE ANALOGUES IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA.

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Despite many therapeutic regimens introduced recently, CLL is still an incurable disorder. The symptomatic disease can be effectively treated with purine analogues, glucocorticoids, alkylating agents, monoclonal antibodies or combination of these. Some patients, however, with relapsed or refractory disease, have limited therapeutic options. Thus there is an urgent need to discover a novel, less toxic and more effective drugs for CLL patients. Among other options, the use of immunomodulatory drugs or plant-derived substances were reported to improve results of CLL treatment. Resveratrol (trans-3,4',5-trihyrdoxystilbene) is a natural compound present in various plant species and relatively abundant in vine. It has many biological and pharmaceutical properties. Among them the anticancer properties of this naturally occurring substance were widely studied in several human cancer cells including B-cell malignancies. In this study, we attempted to explore whether resveratrol can induce apoptosis of CLL leukemic cells in peripheral blood and bone marrow microenvironments and to assess interactions between resveratrol and two purine analogues - fludarabine and cladribine in terms of their effect on apoptosis of CLL cells. The experiments were performed in 24 h cell cultures of peripheral blood and bone marrow obtained from 25 untreated CLL patients. The cultures were supplemented with resveratrol and resveratrol in combination with cladribine or fludarabine. We analysed the percentage of $CD19^+/CD5^+$ leukemic cells with active caspase 3 expression as a marker of apoptosis with the use of multi-colour flow cytometry technique. Results of our study revealed that resveratrol induced apoptosis of CLL leukemic cells. The percentage of cells with caspase 3 expression in 24 h cultures with resveratrol was significantly higher than the level of spontaneous apoptosis seen in the untreated 24 h parallel control cultures. Such an increase in frequency of resveratrol-induced apoptosis was observed as far as peripheral blood and bone marrow cultures were concerned (18.4±9.3 *vs.* 23.3±9.1 and 19.2±8.4 *vs.* 27.8±10.4), the percentage of apoptotic cells was significantly higher in bone marrow than in peripheral blood (with P=0.006). The frequency of apoptosis in resveratrol+fludarabine treated cultures was significantly higher than in resveratrol treated cultures (28.1±9.2 *vs.* 23.3±9.1, P=0.0003) also than in fludarabine-treated cultures (28.1±9.2 *vs.* 22.3±9.2, P=0.0004). Similarly, a significant increase in apoptosis was observed in resveratrol+cladribine-treated cultures in comparison to both resveratrol cultures and cladribine cultures (32.1±13.3 *vs.* 23.3±9.1, P=0.0004 and 32.1±13.3 *vs.* 25.9±9.1, P= 0.0004, respectively). This pro-apoptotic effect was synergic for resveratrol and fludarabine as well as for resveratrol and cladribine. The obtained results indicate that resveratrol can be used in the treatment of CLL patients as a single agent as well as in combination with purine analogues. This naturally occurring substance may be a good therapeutic option for CLL patients, especially older ones in whom there are some limitations in using aggressive treatment. On the other hand there is a possibility of lowering purine analogues dose used in combination with resveratrol bbecause of their synergic effect.

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MUTATION STATUS AND PREFERRED UTILIZATION OF IGVH GENE IN CHRONIC LYMPHOCYTIC LEUKEMIA AND ASSOCIATIONS WITH COMMON BIOLOGIC ABNORMALITIES

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Background. Chronic lymphocytic leukemia (CLL) is a clinically heterogeneous disease whose outcome can be partially predicted by the mutational status of immunoglobulin heavy chain variable (IgVH) genes. Moreover, relationships of various clinical manifestations have been reported with specific IgVH genes identified in leukemic cells. Here we investigated the associations between usage of specific IgVH genes and clinical features in CLL. *Aims.* To further characterize the biological features of this disease, we performed IgVH gene mutational status in 231 cases of CLL for gene family preferences and biases, and investigated the mutation status of VH genes against common biologic features of patients and immunophenotype of CLL. *Methods.* cDNA was reversely transcribed from the total RNA extracted from the peripheral blood from consented CLL patients. Polymerase chain reaction (PCR) and cycle sequencing were performed, and analyzed by a Genetic Analyzer. The percentage divergence at the IgVH loci was obtained by matching the resultant sequences to the NCBI Immunoglobulin data base (http://www.ncbi.nlm.nih.gov/igblast). *Results.* The most common gene families were IgVH 1 (26.2%), 2 (2.6%), 3 (45%), 4, (21%), 5 (3.0%), 7 (1.3%), and 9 (0.9%). The most common specific gene above 10% of total events was IgVH 1-69 (16.2% of the total), followed by 3-30 (7.9%), 4-34 (5.7%), 3-23 (5.2%), 4-39(4.8%), 4-59 (4.8%), and 3-7 (4.4%). Somatic hypermutations (>=2%) were present in 46%, and hypomutation (<2%) in 54% of the total CLL. The somatic mutations didn't occur uniformly among subgroups. The hierarchy of hypomutations occured in the order of IgVH 1-69 (27.4%)> 3-30 (6.5%)> 3-48 (5.6%)> 1-3 (4.8%)> 4-39 and 4-59 (4%), and hypermutation as IgVH 4-34 and 3-23 (10.5%)> 3-30 (9.5%) > 3-7(6.7%)> 4-39 and 4-59 (5.7%). We compared the mutation status of HV genes against selected other known biologic features. Positive associations were present between hypomutation and female patients (RR 1.36, 95% CI 1.03-1.80, P<.05), CD38 (>5%) expression (RR 2.36, .95 CI 1.64-3.40), (del)11q22.3 (RR 4.79, 95% CI 2.09-10.98, P<.0001), trisomy 12 (RR 1.84, .95 CI 1.02-3.33) and complex karyotypes (RR 1.31, 95% CI 1.06-1.61, P<.01). The hypermutation status of HV gene was positively associated with 13q- (OR 4.38, 95% CI 2.52-2.7.62, P<.0001). There was no significant association of hypomutation with age. *Conclusion.* Our results confirmed and extended the observations of a biased utilization of HV genes among B-CLL patients. The gene most preferentially used was 1-69 (16.2%) and 92% of CLL patients with this gene were hypomutated. The second most frequently used gene was 3-30, which had no preference to mutation status (55.6% mutated *vs.* 45% unmutated). IgVH 4-34 and 3-23 were frequently seen after IgVH 1-69 and 3-30, and associated with hypermutation, 84.6% and 92%, respectively. The IgVH 4-34/DP 63, encoding an antibody that recognizes autologous determinants of RBC, occured in 5.6% and was one of the most common HV genes with hypermutation (10.5%). The extended studies correlated hypomutation of IgVH to chromosomal abnormalities with prognostic potential such as 11q22.3-, +12, also to female patients, and CD38 overexpression (>5%), while hypermutation correlated to 13q-.

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DYNAMIC CHANGE OF SERUM THYMIDINE KINASE LEVELS IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKAEMIA

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Background. Thymidine kinase (TK) is an important enzyme, which reflects cell division and DNA activity. It is known that TK levels are increased in acute leukosis, lymphomas and myeloma . However, nothing is stated about the level of TK in chronic lymphocytic leukemia (CLL). *Aims.* Serum TK levels in newly diagnosed patients with CLL was determined both pre treatment, 6 months and 12 months post initiation of chemo and glucocorticoid therapy. Patients were grouped using the Rai staging system and serum TK levels assessed within each stage. We try to utilize this marker as a prognostic factor in this disease will improve the appropriate selection of more effective treatment. *Methods.* The research group consisted of 120 newly diagnosed CLL patients during the period 2007 to 2009. Since levels of serum TK are low, it is best to use a method based on enzymic activity. In our study the following ELISA technique was used. Serum TK levels in healthy donors is less then 50 ng/L. Latvian Central Ethics Commission approved the research design and all patients enrolled in this investigation had given the informed consent. *Results.* 120 patients were assessed in this study. The patients were divided according to Rai staging system as follows: 7 patients in 0 stage, 38 patients- I stage, 40 - II stage, 19 patients in III stage, and 16 -in IV stage. Serum TK levels was determined as the normal or above the normal (> 0 ng/L) in all patients. Treatment with Chlorambucil had 50 patients. Fludarabinum/Prednisone - 24 patients. COP regimen - 17, mini CHOP regimen - 4, Fludarabinum/Cyclophosphomide - 7 patients, without any therapy - 18 patients. The results showed that 6 months post initiation of therapy serum TK levels were decreased in all treatment groups. (2821±375 ng/L pre therapy, 1557±214 ng/L post therapy P=0.0001)) The 12 month post initiation of therapy group showed a decrease in serum TK levels however the statistical probability was lower than for the 6 month group (2821±375 ng/L pre therapy, 1775±312 ng/L post therapy, P=0.02). We demonstrated a slight positive correlation between the level of serum TK and Rai stage of disease after analysis of all cases (r= + 0,36; P<0.0001). *Conclusions.* TK is increased in the serum of all newly diagnosed CLL patients. Serum TK levels are reduced after initiation of therapy, A highly significant reduction in serum TK levels is seen after 6 months post initiation of therapy (P= 0.0001) however after 12 months post initiation of therapy levels still showed a reduction but not as significant as after 6 months. (P=0.02) When analyzing all patients together, found a weak positive correlation between the level of serum TK and Rai stage (P<0.0001).

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HLA-G EXPRESSION IN B CHRONIC LYMPHOCYTIC LEUKEMIA

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B cell chronic lymphocytic leukemia (B-CLL) is the most common adult leukemia in Western countries. It is charactarized by an accumulation of long-lived, functionally inactive, mature-apearing neoplastic B lymphocytes. B-CLL has a variable clinical course. Some patients have an exellent prognosis never requiring treatment, whereas in others the survival is short despite early initiation of therapy. Classical Rai and Binet staging of CLL is being superseded by new prognostic markers. In the last few years, it has been suggested that the involvement of human leukocyte antigen-G (HLA-G) in several tumoral processes and its likely participation as a factor of immune tolerance in malignant cells. Recent studies indicate an ectopic up-regulation in tumor cells that may favor their escape from anti-tumor immune responses. The role of HLA-G in B-CLL has not been defined. For this report, 28 patients suffering from B-CLL were analyzed for the expression of HLA-G by flow cytometry using the anti-HLA-G spesific monoclonal antibody MEM/G9 and its correlation with other prognostic factors, RAI stages, β2-microglobulin, ZAP-70 protein and CD38 ekpression level. We observed low expession of HLA-G on leukemic B cells (1 to 22%). We detected no correlation with Binet staging , CD38 and ZAP-70 .In comparison with the We further observed that the patient group with low HLA-G expression (<10%) had significantly lower levels of IgG than the group with high level of HLA-G (>10%). Hence the 23 patients out of 28 have been followed up without any treatment, the correlation of the progression free survival time evaluation is stil under study.

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ROLE OF SECOND GENERATION TYROSINE KINASE INHIBITORS IN OSTEOBLASTOGENESIS

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Backgrounds. The BCR-ABL inhibitor imatinib is standard first-line therapy for patients with chronic myeloid leukemia (CML) and has revolutionized treatment of the disease. However, it has been demonstrated that long term treatment of CML patients with IM is associated with altered bone and mineral metabolism with reduction of bone remodeling and increase of bone mineral density. In this perspective, we and others have demonstrated that *in vitro* IM treatment can increase the capacity of hBM-MSCs to differentiate into osteogenic lineage and favour osteoblastogenesis. The mechanisms that are responsible for this effect are not fully understood but an inhibition of PDGF-R_ (Platelet Derived Growth Factor- Receptor beta) axis has been suspected on the basis of some *in vitro* evidences. However, it is not clear whether the effect of IM on osteoblastogenesis may be entirely due to inhibition of PDGF-R signaling. In order to evaluate if stimulation of osteoblastogenesis is a common feature of other tyrosine kinase inhibitors (TKI) approved for the treatment of patients with CML, we evaluate the osteoblatic differentiation of Mesenchymal Stem Cells derived from bone marrow (BM-MSCs) after *in vitro* treatment with Dasatinib (DA) or Nilotinib (NI) or Bosutinib (BO). *Methods.* Mesenchymal stem cells (hBM-MSCs) were obtained from bone marrow samples of normal healthy adults after informed consent, isolated by density gradient (mononuclear fraction) and cultured in standard medium (SM). hBM-MSCs was induced to differentiate in osteoblastic cells by treatment with osteogenic medium (0.2 mM ascorbic acid, 0.1 μm dexamethasone and 10 mM β-glycerophosphate) (OM) with or without DA 2nM or NI 100 nM or BO 5nM. Expression of osteoblast-associated genes such as osteocalcin (OCN), RUNX2 and Bone morphogenetic protein (BMP-2) were evaluated by reverse transcription-polymerase chain reaction (RT-PCR) at 21 days of culture. *Results.* We found that the addition of DA and to a greater extend NI induced expression of osteogenic markers mRNA as compared to cultures with SM or OM only. However, treatment with BO did not induce increase of osteogenic markers as compared to controls. Table 1 indicates RT-PCR fold increase of osteogenic markers compared to cells treated with standard medium only. Conclusion. In conclusion, we showed that besides IM, other TKI such as DA and NI, but not BO, increase osteogenic markers (BMP-2, Runx2 and OCN) mRNA expression in BM-MSCs. Since BO differ from the other TKI because of its low affinity to PDGF-R, these experiments indicate that inhibition of PDGF-R is a major pathway in the induction of osteoblastogenesis by TKI. In this perspective, long term therapy with Bosutinib should not induce perturbation of bone and mineral metabolism and it could represent the treatment of choice for children affected by CML.

Table 1.

	Standard medium	Osteogenic medium (OM)	Dasatinib	Nilotinib	Bosutinib	Dasatinib + OM	Nilotinib + OM	Bosutinib + OM
RUNX2	1	17,7 ± 3,4	17,7 ± 3	20,3 ± 2,8	1,1 ± 1,8	16,5 ±2	28,8±4,3	19 ± 1
OCN	1	6,9 ± 2,3	3,7 ± 2	5,6 ± 1,8	1 ± 0,9	9,8 ± 2,2	12,1 ± 1,9	3,7 ± 1
BMP2	1	11,7 ± 2,4	8,8 ± 3,3	7,7 ± 3,6	0,9 ± 1,6	12,1 ± 4	12,5 ± 2,7	10,9 ± 2

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HAIRY CELL LEUKEMIA: RESULTS OF LONG-TERM FOLLOW UP OF 214 PATIENTS

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We followed up 214 patients (pts) with hairy cell leukemia (HCL) sinse 1995 till 2010(15 yrs). The diagnosis was made according to standard criteria, including routine clinical, radiological, sonography data, blood and bone marrow cytology, TRAP, immunophenotyping, trephine and/or splenic morphology with immunohistology. There was 145 man and 69 women (m:f ratio = 2,1:1). Classical HCL had 159 pts, HCL-variant form 55 (26%) pts. Median age was 51 yrs (range 27-81); patients younger than 45 yrs appeared 53 (25%) pts. 203 pts received a-interferon for 8-12 wks (3 mln Un 3 times per every wk sc) with subsequent cladribin standard prescription in 157 pts with achieving complete remissions for median 7 yrs (range 0.6 - 15) in 85% of them. Thus it was possible to avoid agranulositosis in 95% pts while there were two prolonged such events in initial treatment without preliminary a-IF. The response to this treatment was equal either in classical or variant form of HCL. Relapses happened in 33 (21%) pts after median 4 yrs (range 1.3- 12), most of patients had classical form of HCL. It was noted that from 33 relapsed pts 26 (79%) were younger 45 yrs. Died 18 (8%) pts, aged 31-71 yrs at attendance, 14 pts had classical HCL and 4 pts had HCL-variant form, 9 pts were of 31-45 yrs old. The main cause of death in 6 young pts was HCL progression; 4 old pts died from severe infections at admittance, the last 5 pts - from diseases not linked to HCV. Secondary tumors occurred in 10 (5%) pts aged 62 (46-73) yrs. Tumors were discovered before HCL diagnosis in 2 pts, after 11 (range 2-19) yrs following therapy with a-IF in 4 pts; after 4 (range 0.5-10) yrs following cladribin course in 4 pts. Localization of these 10 secondary tumors were: 3 urogenital (uterus, bladder, prostate), 3 gastrointestinal (gastric cancer, colon), 2 lungs,1 breast, and 1 soft tissues. Among them 4 pts died in complete HCL remission. We propose to intensify initial therapy of HCL in patients younger than 45 yrs with subsequent anti-CD20 monoclonal antibodies.

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PROGNOSTIC SIGNIFICANCE OF ABDOMINAL CT SCAN IN CLL PATIENTS IN BINET STAGE A: PRELIMINARY RESULTS OF A PROSPECTIVE, MULTICENTER OBSERVATIONAL-CLL1- GISL STUDY

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Background. The Rai and Binet staging systems represent the backbone for assessing prognosis in CLL patients. However the staging systems are not devoid of some limitations, among these there is the lack of evaluation of abdominal lymphadenopathy. Recently, a retrospective study challenged this notion highlighting the importance of prospective validation of abdominal CT before routine inclusion in CLL work-up. *Aims.* We investigated the clinical significance of abdominal CT in the initial work-up of Binet stage A CLL patients, included in the prospective multicenter O-CLL01 GISL study. *Patients & Methods.* To date, 380 patients have been enrolled in the trial started in April 2007 and abdominal CT were available for 127. Median age was 60.5 years (range, 33-70 years) and 61% of patients were male. Ninety-one patients were at low risk and 36 at intermediate risk by Rai classification. LDH was elevated in 11% of cases and B2-microglobulin in 28%. Thirty-six% of cases were IgVH unmutated, 42.5% had a high ZAP-70 expression, 22% were CD38 positive (>30%). FISH data were available for 116/127 cases; the most frequent abnormality was del(13q14) (46%), followed by trisomy 12 (12%), del(11q22.3) (6%) and del(17p13) (3%), 33% of cases were normal. Cytogenetic abnormalities were clustered in 3 risk groups [i.e. low (del(13q14) and normal), intermediate (trisomy 12) and high risk (del(11q22) and del(17p13)]. Abdominal CT was defined as abnormal when ≥1 anomalous lymphnode regions, including spleen enlargement, were observed. *Results.* Fifty-eight of 127 patients (46%) showed an abnormal abdominal CT. Notably, 78% were male, LDH was elevated in 5% of cases and B2-microglobulin in 38%, 48% were IgHV unmutated, 52% had a high ZAP-70 expression, 38% were CD38 positive and 15% showed a high-risk FISH. Male gender (P=0.001), elevated B2-microglobulin level (P=0.036), high CD38 expression (P<0.0001) and IgHV germline status (P=0.016) correlated with an abnormal abdominal CT. At logistic regression analysis only male gen-

der (P=0.013; HR 3.1, CI 1.3-7.7) and high CD38 expression (P=0.008; HR 5.1, CI 1.5-17) remained independently associated with abnormal abdominal CT. Time to progression was significantly shorter in patients with an abnormal CT than in those with a normal CT (2-years TTP probability, 86% *vs.* 100%; P=0.003). According to the Rai classification 35/91 (38.5%) low risk patients showed an abnormal abdominal CT. In this subset of patients male gender and high CD38 expression again correlated with an abnormal abdominal CT at both univariate (P<0.008 and P<0.0001, respectively) and multivariate analysis (P=0.016; HR 3.6, CI 1.3-10.3, P=0.002; HR 6.6, CI 2-21.8, respectively). Patients with an abnormal CT showed a TTP significantly shorter than those with a normal CT (2-years TTP probability, 82.5% *vs.* 100%; P=0.015). *Conclusion.* Our data indicate that abdominal CT allow discrimination among Binet A CLL patients 46% of cases with abdominal lymphadenopathy or splenomegaly. Moreover abnormal CT were associated with bad prognostic parameters as male gender and high CD38 expression. Taken together, abnormal abdominal CT predict disease progression. Similar data are observed in Rai stage 0 patients. Thus the inclusion of abdominal CT in the initial work-up of patients in early stages can provide relevant clinical information.

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ENLARGED ABDOMINAL LYMPH NODES AND EARLY RESPONSE ARE NEW FAVORABLE PROGNOSTIC MARKERS IN CLL PATIENTS TREATED WITH FLUDARABIN-CONTAINING REGIMENS

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Background. Initial prognosis estimation in CLL can be done by clinical staging (Rai and Binet systems) and a couple of laboratory methods, which are rather labour and time consuming. E. Montserrat *et al.*. showed that CT scans can be used for prognosis prediction. In particular presence of abdominal lymphadenopathy was associated with inferior survival in Rai 0 CLL pts. Fludarabine-containing regimens are widely used as first-line treatment in CLL. We sought to investigate whether interim response after three cycles of such therapy interferes with post-treatment OS and PFS. *Aims.* To evaluate prognostic significance of abdominal lymph nodes enlargement (EALN) and response after three cycles of therapy in CLL pts receiving fludarabine-containing regimens (FC and FCR). *Methods.* 75 untreated CLL pts were included according to intent to treatment by fludarabine containing regimens (41 assigned to FC, 34 to FCR). Informed consent was obtained in each case. Treatment schedules were standard. Lymph nodes were revealed by CT and ultrasound before therapy (available in 71 pts). Lymph nodes >10 mm in diameter were considered abnormal and >100 mm in diameter - bulky. Early response (ER) was defined as PR and CR after 3 cycles of therapy, others were considered non-early responders (nER). CD38 was measured by cytoflow with 30% cut off (available in 57 pts). VH genes were analyzed by cDNA sequencing with 98% threshold (available in 32 pts). CR was defined according to NCI criteria. Statistics were calculated in SPSS17. Cox regression was used for HR estimation. Model included regimen, stage, CD38 status, presence of EALN and ER.

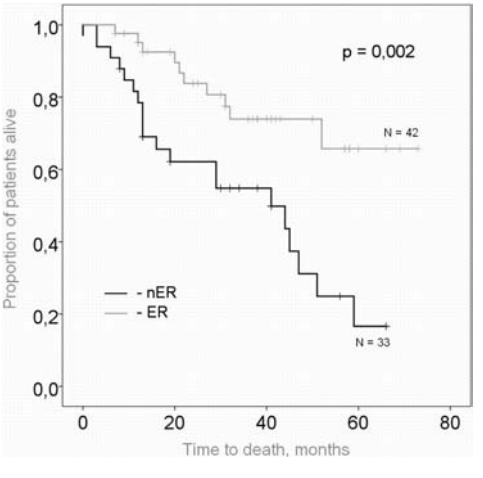

Figure. Overall survival in ER *vs.* nER.

Results. There were 42 pts in ER group and 33 nER. ER pts in comparison to nER had higher median PFS in both FC (28 *vs.* 14 mo., P=0,001) and FCR (58 *vs.* 18 mos., P=0,018) arms. ER pts with standard adverse prognostic factors did better than nER with similar prognostic factors (median PFS in CD38-pos 31 *vs.* 12 mos. P=0,05; UM-CLL 32 *vs.* 18 mos. P=0,03; Stage C 31 *vs.* 9 mos, P=0,001). OS was significantly higher in ER (median OS n.r. *vs.* 41 mos, P=0,002). EALN were present in 41 pts (58%), 16 pts had bulky EAL (23%). PFS was lower in the group of pts with EAL in comparison to pts without EAL (18 *vs.* 48 mos, P=0,001). Difference in PFS has been also revealed in FC and FCR subgroups (16 *vs.* 25 mos, P=0,003 for FC; 22 *vs.* 56 mos, P=0,01 for FCR). Most of the pts with EALN did not reach ER (34,15% *vs.* 89,65% in EALN-group, P<0,01). Interestingly most of the pts with EALN had UM-CLL as opposed to non-EALN group (83,3% *vs.* 43,75%, P=0,054). In Cox regression analysis only presence of EALN and absence of response after three cycles of therapy were significantly associated with decreased PFS (HR 2,679, P=0,013, CI 1,229-5,842 and HR 2,334, P=0,05, CI 0,989-5,513 respectively). *Conclusion.* Presence of EALN appears to be an independent adverse prognostic factor in CLL associated with decreased PFS. Early responding CLL pts form a specific cohort with extremely favorable prognosis.

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CHRONIC LYMPHOCYTIC LEUKAEMIA PATIENTS WITH V1-69 GENE REARRANGEMENT HAVE AN UNFAVOURABLE CLINICAL OUTCOME

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Background. Mutational status of the immunoglobulin heavy chain variable region (IgVH) gene is a robust and independent predictive factor in patients with B-cell chronic lymphocytic leukaemia (B-CLL). The V1-69 gene is the most frequently rearranged V(H) gene and is almost always unmutated. Nevertheless, the prognostic role of V1-69 gene rearrangement is not clear and the published studies have reported controversial results. *Aim.* To analyze the haematological features, clinical behaviour and outcome of patients with V1-69 gene rearrangement. *Methods.* IgVH mutational status was analyzed in 328 patients with B-CLL who met the NCI-WG diagnostic criteria. IgVH mutational status was analyzed using cDNA transcribed from B-CLL RNA for touch-down reverse transcription polymerase chain reaction (RT-PCR) with degenerate primers for VH1-7 families; IgVH were aligned to the nearest germline using the Ig BLAST database. A sequence homology cutoff of 98% was used to define the IgVH mutational status. The V1-69 gene was clonally rearranged in 44 (13.5%) of 328 cases. All but one (97.7%) patient had unmutated IgVH. Most of the unmutated cases (77.2%) had 100% sequence homology with the nearest germline V(H) gene. The median age at diagnosis was 60 years (36-83) and the M/F ratio was 3.4:1. Binet clinical stages were as follows: A 14%, B 20% and C 10%. FISH analysis was performed in 39 of 44 patients (88.6%); high-risk genetic abnormality (17p, 11q or 6q deletion) was detected in 22 (56%) cases. ZAP-70 was evaluated in 26 of 42 patients - all of the cases were positive. The immunophenotypic profile was aberrant in 18 patients (42.8%). Treatment was initiated in 36/44 patients (82%), with first-line therapy comprising chlorambucil (5/36), fludarabine-based or RFC regimen (26/36) or R-C(H)OP-like regimen (5/36). *Results.* Complete response was achieved in 5 (15%) and partial response in 19 (56%) of 34 patients; the overall response rate after first-line therapy reached 72%. After a median follow-up of 51 months, 17 (38.6%) of 44 patients died. The median overall survival (OS) reached 91.6 months (95% CI 78.4-104.8); the median treatment-free survival (TFS) was 58.0 months (95% CI 40.1-76.0). Both OS and TFS were not significantly different in patients with or without high-risk cytogenetic abnormality (P=0.32 and P=0.09, respectively). Similarly, Binet stage did not influence OS and TFS (P=0.1 and P=0.2, respectively). *Summary.* Patients with V1-69 gene rearrangement almost always have unmutated IgVH, all of the examined patients were ZAP-70 positive and more than half of the patients had a high-risk genetic abnormality. The survival and treatment initiation are not influenced by unfavourable cytogenetics or Binet stage. The overall response to initial therapy is unsatisfactory. The survival is uniformly worse than the published results in unselected patients with unmutated IgVH. Generally, the outcome of the population with V1-69 gene rearrangement is unsatisfying due to an accumulation of unfavourable prognostic factors. *Acknowledgements.* Supported by the grants of the Ministry of Edu-

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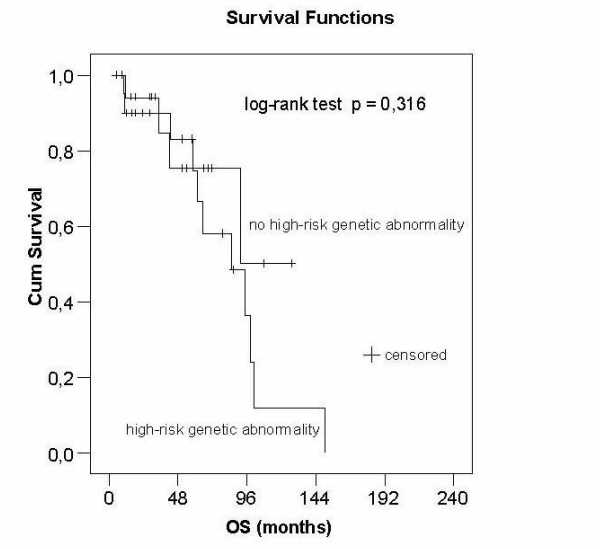

Figure 1.

1282

THE USE OF GRANULOCYTE COLONY-STIMULATING FACTOR (G-CSF) IN CLL PATIENTS TREATED WITH FLUDARABINE, CYCLOPHOSPHAMIDE AND RITUXIMAB, (FCR) IS ASSOCIATED WITH LONGER PROGRESSION FREE SURVIVAL (PFS)

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Background. Chemoimmunotherapy with fludarabine, cyclophosphamide and rituximab, (FCR) is currently the preferred first or second line treatment for fit patients with chronic lymphocytic leukemia (CLL).With this treatment high overall remission rates of 70 to 90% (CR 25 to 44%) are achieved. Severe neutropenia (CTC grade 3+4: 42 to 62%) and infections (5 to 18%) are the major side effects. Currently, prophylactic use of G-CSF is not recommended for FCR treatment in clinical studies and data on G-CSF are not reported. *Patients & Methods.* We retrospectively analysed the use and impact of G-CSF in a cohort of 32 unselected, consecutive patients with CLL and related entities treated with FCR between 2004 and 2008 in a single center. Twenty-five patients (78,1%) were previously untreated. Patients were older than published cohorts (median age 66 years, range 50 to 82 years, 23 males and 9 females). G-CSF was used for treatment of febrile neutropenia (FN) or infection. G-CSF was also given, when CTC grade 3 neutropenia (ANC 500-1000/uL) occurred, but not for primary prophylaxis. Twenty-three patients (71,8%) received antibiotic prophylaxis with sulfomethoxazole/ trimethoprim. *Results.* The 32 patients received 156 cycles of FCR. Two patients (5%) had febrile neutropenia. CTC grade 3 and 4 neutropenias (ANC 0-500/uL) were observed in 16 patients (50%). Neutropenia predominantly occurred in FCR cycles 1 and 2. G-CSF was administered in 38 (24,6%) of all 156 cycles. The median dose of G-CSF per patient (n=16) was 75 million units (30 to 1.110 million units). Dose reductions for patients receiving G-CSF were recorded in 34 cycles, for patients without G-CSF dose reductions were reported in 48 cycles. Dose reductions were performed according to the rules of the GCLLSG. Dose delays were seen in 10 patients (7 patients with G-CSF and 3 patients without G-CSF). The number of cycles completed in comparison to planned cycles was not different in patients with or without GCSF (patients with G-CSF: 81%, patients without G-CSF 79%). Only 3 patients (7.5%) were hospitalized due to infection or FN (2 before and 1 patient without FN, infection only) currently, 27 of 32 patients are still alive, 5 patients died. The overall response rate in all patients was 84,4% (53% CR). For CLL patients receiving G-CSF the ORR was 93,8% and for patients without G-CSF 75 %. Progression free survival (PFS): only two patients in the G-CSF group had progression. Median PFS at 24 months was 93.75% in patient with G-CSF and 37,5% in patients without G-CSF. (P=0.0001) Overall survival in patients with G-CSF was 93,75% and for patients without G-CSF was 75%. (P=0.221). *Conclusions.* Data suggest that routine use of G-CSF not only to prevent FN but also to increase density may have a positive effect in CLL. Interestingly patients receiving G-CSF had a significantly longer progression free survival (Figure 1). Patients receiving G-CSF have better outcome, progression free survival than patients without G-CSF. Those results warrant a randomized clinical trial of prophylactic use of G-CSF to increase dose densities.

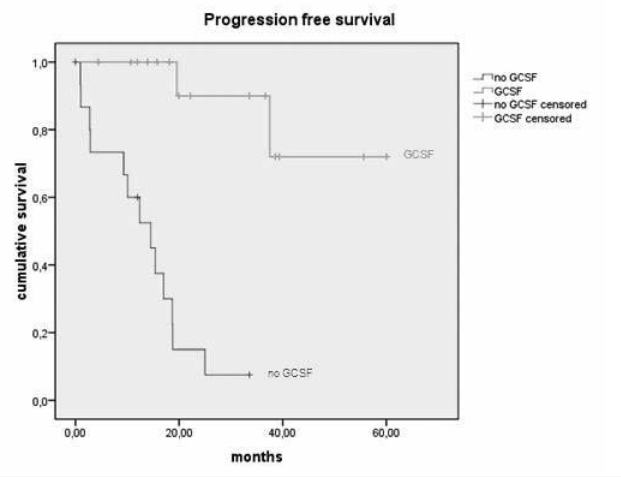

Figure 1. Progression free survival p-value<0.0001.

1283

NON-MALIGNANT B-CELL SUBSETS AS BIOMARKERS FOR THE RISK OF INFECTIONS IN CHEMOTHERAPY -NAÏVE CLL PATIENTS

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Background. Individuals with anatomical or functional asplenia suffer from recurrent infections by encapsulated bacteria (Streptococcus pneumoniae, Haemophilus inluenzae). The impaired immune response to these bacteria is due to the absence of IgM^+ memory B cells, which require the spleen for their generation and/or survival. *Aims.* We here sought to investigate, whether the high incidence of recurrent respiratory infections observed in a subgroup of CLL patients is related to a state of functional asplenia. *Methods.* Sixty-eight patients with chemotherapy-naïve CLL were enrolled in a pilot study. Evaluations consisted of standard laboratory and clinical parameters including type and frequency of infections. Peripheral blood cells were analyzed by multi-parameter flow cytometry. The $CD19^+CD5^-$, non-malignant B cell compartment within the lymphogate was further subdivided by staining for surface IgD and CD27 to discriminate between naïve, marginal zone (MZ) and class switched memory (SM) B-cells, respectively. *Results.* Low numbers (<10 μL) of SM B-lymphocytes were significantly associated with the occurrence of infections (P=0.023; 23/29 *vs.* 20/39), higher age (P=0.0026; 70.7±8.4 *vs.* 63.1±10.7 years), lower leukocyte counts (P=0.041; 28421±21627 *vs.* 52031±58071 μL) and low CD38 expression levels (P=0.0082; 8.7±17.6 *vs.* 26.1±29.6 % CD38+; and P=0.0047; 2/27 *vs.* 15/39 for $CD38^+$ >30%) but not with mutational status or cytogenetic aberrations. Moreover, the occurrence of infections per se was significantly correlated with higher age (P=0.047; 68.3±10.8 *vs.* 63.1±9.0 years), lower hemoglobin levels (P=0.032; 13.4±1.6 *vs.* 14.2±1.3 g/dL) and elevated serum beta2-microglobulin levels (P=0.031; 2.6±1.7 *vs.* 1.8±0.8 mg/L). *Conclusion.* Monitoring of residual, non-malignant CD5-B-lymphocyte subsets in the peripheral blood of chemotherapy-naive CLL patients may represent a novel biomarker to identify CLL patients at risk for frequent respiratory infections. A prospective study relating the above findings with patient morbidity clearly is warranted.

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EVALUATION OF MINIMAL RESIDUAL DISEASE IN PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA BY MULTIPARAMETRIC FLOW CYTOMETRY AND REAL-TIME PCR

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Background. Chronic lymphocytic leukemia (CLL) is the most widespread type of leukemia in adults. Despite significant progress in treatment, chronic lymphocytic leukaemia remains incurable disease. Aim of the therapy in most patients is to achieve a complete remission (CR) of the disease, which is defined by the NCI-WG criteria ("National Cancer Institute - Sponsored Working Group") and is associated with eradication of majority of tumor cells. CR with negative MRD is associated with favorable extension of the remission period, because residual leukemic cells can cause a relapse of the disease. Methods of multicolor flow cytometry and polymerase chain reaction (PCR) enable to detect minimal residual disease (MRD) in patients with CLL, who achieved CR. *Aims.* We have implemented standardized multiparametric flow cytometry protocol for the detection of MRD in CLL (modified according to A. Rawstron, 2007). MRD was also detected by molecular biology approaches with the detection of IgVH specific clones with sensitivity 1:106 cells. Both methods we compared in the group of patients with CLL. *Methods.* A group of 17 patients with diagnosis of CLL, who achieved CR after alogeneic transplantation of peripheral blood stem cells (PBSCT), were included into the observation. Samples of peripheral blood and bone marrow of patients were repeatedly analyzed by flow cytometry. Briefly, samples were analyzed if $CD5^+19^+$ cells represented less then 5% of leukocytes. Samples were incubated with following antibodies directly labeled with fluorochromes FITC, R-PE, PE-Cy5 and PE-Cy7: anti- CD5, CD19, CD20, CD22, CD38, CD43, CD79b, CD81 (Invitrogen, U.S.A.). CD5 and CD19 antibodies were included in each test tube. Erythrocytes were eliminated by hypoosmotic hemolysis according to the standard procedure and samples were analyzed on flow cytometer Cytomics FC500 (Beckman-Coulter, U.S.A.). MRD calculation was performed at web calculator (www.mrd-cll.org). Detection of clonal IgVH was performed by RQ-PCR with LNA probe (Locked Nucleic Acid, TaqMan technology). Universal LNA modified TaqMan probes (TIB MolBiol, Germany) specific for all clones of each VH family were designed to FR3 region. Specific oligonucleotides were designed for each patient, one of those always localized to clonotypic region. Plasmid with cloned patient specific IgVH sequence was prepared for quantification. Albumin was selected as a reference gene. Sensitivity of the method reached 1: 105-106. There were no problems with non-specific amplification (modified protocol, Brüggemann *et al.,* 2000). *Results.* In the cohort 17 of patients who underwent alogeneic PBSCT, 12 of them achieved MRD-negative CR. Five patients had detectable MRD after PBSCT. 11 of these patients were simultaneously evaluated for MRD by both methods. There was a very good correlation between flow cytometry and PCR approaches (r=0,8, in paired samples r=0,93). *Conclusions.* Our results suggest that high-sensitivity flow cytometry method of MRD detection in CLL is applicable to peripheral blood and bone marrow samples of patients after PBSCT and that it is comparable to RQ-PCR technique. Evaluation of MRD in patients with CLL after therapy by high-sensitivity methods has a prognostic importance. It also allows for early detection of relapse and early change of the treatment strategy.*Acknowledgements.* This work was supported by grant IGA MZ CR No. NR9671-4 and MSM 021622430.

1285

DETECTION OF HAIRY CELLS (HC) IN THE BLOOD IS NOT SUFFICIENT TO PREDICT LONG TERM RELAPSE IN HAIRY CELL LEUKAEMIA (HCL)

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Background. HCL is a lymphoproliferative B-cell malignancy which represents about 2% of all adult leukemia. Purine analogues have transformed its prognosis with a median relapse free survival of 16 years. Overall survival 15 years after the first treatment reaches 95% with pentostatin and 100% with cladribine. However, early relapse may occur with emphazise the need to define new criteria to identify those patients. *Aims.* Detection of HC in the peripheral blood (PB) by flow cytometry at complete remission (CR) as criteria of relapse. *Methods.* Retrospective study of HCL patients treated between 2000 to 2010 with either pentostatin or cladribine. Response evaluation was performed with criteria of the Consensus Resolution and safety evaluation with NCI criteria. Immunophenotyping was performed on peripheral blood using a 6-color panel of antibodies (Ab) with the following combinations: CD43/CD103/CD19/CD25/CD11c/CD45 and IgG/IgG/CD19/IgG/IgG/CD45/. Fifty to one hundred µL of blood was incubated with the different Abs for 10 minutes at room temperature. Red cells were lysed with FACSTM Lysing Solution (BD), then cells were washed and resuspended before analysis on a 6-color flow cytometer . CD19 positive B cells were gated. Hairy cells were identified on the basis of bright coexpression of CD11c and CD103, positivity for CD25 and negativity for CD43. Between 32500 and 235000 cells were routinely acquired . The statistical test Chi-square was used. Kaplan-Meier survival curves were compared using Log-Rank test. *Results.* Twenty two patients were treated and followed between 2000 to 2010. Sisteen patients received pentostatin 4mg/m^2 IV every 2 weeks until maximum response, consolidated with two further doses. Six patients received cladribine by continuous IV infusion (0.1 mg/kg/day) over 7d. A repeated cycle of cladribine was given if CR was not attained after the first cycle. The CR rate was 100%. There was no safety significant difference between cladribine and pentostatin treatment on the following parameters. Regarding infections, we observed 21% grade 1/2 for pentostatin arm; and 19% grade 1/2, 5% grade 3/4 for cladribine arm (P=0.0915). Concerning hepatic toxicity, we detected 5% grade 1/2, 5% grade 3/4 for pentostatin arm; and 0% for cladribine arm (P=0.4416). For skin toxicity, 14% grade 1/2 for pentostatin arm; and 5% grade 1/2 for cladribine arm (P=0.7079) occurred. There was no digestive toxicity . At a median follow up of 2 years, median event free survival (EFS) was 91% without any significant difference between cladribine and pentostatin treatment (94% *vs.* 83%, P=0.7055). Nine patients were evaluable in the PB by flow cytometry. Median hairy cells count was 198 (range 0-24900). Five on nine patients (56%) had a detectable disease, but none of these patients have relapsed to date. *Summary.* Cladribine and Pentostatin are safety treatments for HCL. This disease has a favorable outcome with these treatments but some early relapse occurs. Immunophenotyping evaluation at CR may still detect hairy cells but the correlation with relapse is unclear. It is therefore necessary to validate a cut-off in prospective study to predict the relapse.

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LOW DOSE ORAL FLUDARABINE PLUS CYCLOPHOSPHAMIDE IN ELDERLY PATIENTS WITH UNTREATED AND REFRACTORY CHRONIC LYMPHOCYTIC LEUKEMIA (RETROSPECTIVE STUDY)

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Background. Fludarabine plus Cyclophosphamide (FC) at convetional doses is highly effective in Chronic Lymphocytic Leukemia (CLL) and is currently indicated in first line therapy. Oral FC at reduced doses remains highly effective in elderly patients with low-grade non Hodgkin lymphomas other than CLL. *Aims.* To test tolerability and efficacy of oral FC at reduced doses in patients unfit for standard regimens. *Methods.* Thrirty-eight elderly patients with untreated (UT-CLL, n=21) or refractory CLL (R-CLL, n= 17), requiring treatment according tp 1996-NCI criteria, were given oral F (25 mg/m^2/day) and C (120 mg/m^2/day) in an outpatient regimen for 4 consecutive days every 4 weeks for a maximum of 4 cycles. Median age of the whole population was 65

years (range 60-75). Performance status was ≥ 1 in 72% patients. Median time from diagnosis or from prior treatmetn to FC was 21. months (range 1-61). Patients were evaluated after every cycle for toxicity and hematological response. Toxicity and responses were defined according to NCI criteria, with the exception of not repeating bone marrow aspirate after treatment termination in a few cases. Progression, new treatment or death was defined as "event". *Results.* Patients received median 4 cycles (5 in UT-CLL, 4 in R-CLL). 10 patients reduced the number of cycles because of fatigue (1 patient), heart failure (1), idiopathic thrombocytopenic purpura (1) in the UT-CLL (3/21) or infection (2), prolonged isolated thrombocytopenia or pancitopenia (2) and idiopathic thrombocytopenic purpura (2) in R-CLL (6/17). Overall, 35/38 (92,1%) patients obtained a response (15/38 CR, 39,4%; 20/35 PR, 52,6%). Among UT-CLL, all responded to treatment (10/21 CR, 47,6%; 12/21 PR, 57,1%). Among R-CLL, 10/12 (83,5%) responded (5/17 CR, 29,4%; 9/17 PR, 52,9;% and 3/17 NR, 17,6%). With a median follow-up of 20 months, 19/35 patients (54,2%) were event-free and 34/38 (89,4%) were alive. Amoung UT-CLL (median follow-up 12 months) 17/21 (80,9%) were event-free and all were alive. Among R-CLL (median follow-up 23 months), 7/17 (41,1%) were event-free and 14/17 (82,3%) were alive. Deaths occurred in the 2R-CLL that had not responded to treatment, after 2 and 3 years, respectively. *Conclusions.* Low-dose oral FC treatment showed good effciacy both in untreated and refractory/ relapsed CLL. The treatment may be useful in elderly patients who cannot benefit of more aggressive or eradicating strategies and is easy to administer on an outpatient basis.

1287

BENDAMUSTINE IN ASSOCIATION WITH RITUXIMAB AS SALVAGE THERAPY FOR SPLENIC MARGINAL ZONE LYMPHOMA

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Background. At present, a prospectively validated treatment for SMZL is lacking. Despite this fact, splenectomy is still deemed to be the best choice for patients requiring treatment. Nevertheless, the high fraction of patients achieving a clinical response following splenectomy eventually relapses or progresses. For such patients, as well as for those who are unfit for splenectomy or unwilling to undergo surgery, systemic treatment may be appropriate. Bendamustine (B) is a purine analogue/alkylator hybrid agent with a unique mechanism of action. Bendamustine has shown a considerable degree of activity as single agent in patients with lymphoid malignancies and there is evidence that its *in vitro* cytotoxic effect may be enhanced by the monoclonal anti-CD20 antibody Rituximab (R). In the setting of several haematological malignancies, the association R-B has proved to be effective and well tolerated. *Purpose.* To asses response rate and toxicity of the R-B association in pre-treated SMZL patients. *Material and Methods.* Clinical data of SMZL patients who received the R-B association as salvage therapy were retrieved. The schedule and dosage of BR was as follows: Bendamustine 80-90 mg/m^2 i.v. days 1-2 and Rituximab 375 mg/m^2 day 1, q28. Presenting features, response rates and toxicities were analysed. *Results.* Six SMZL patients, median age 69 yr (rage 62-82), relapsed after a median of 2 lines of therapy (range 1-3) received R-B treatment. Previous treatments included splenectomy (3 patients), anthracyclines and alkilating agents (all patients), purine-analogues (2 patients), and Rituximab (2 patients). All patients had active disease with peripheral cytopenia and three of them had also B-symptoms and/or symptoms related to huge splenomegaly (spleen tip 7-18 cm below left costal margin). After a median of 4 cycles of therapy (range 2-6), 5 patients attained a CR and one patient, who achieved a complete regression of splenomegaly and bone marrow infiltration, and a 75% reduction of the IgG-k serum monoclonal component, was considered in PR. Haematological toxicity > grade 3 CTC was recorded in two patients (HB: G4; PLT: G3; Neutrophils G4; infection G3). One case of pseudomonas areuginosa bacteremia and one case of febrile neutropenia were recorded in two non-splenectomised patients and both patients recovered after appropriate antibiotic therapy. *Conclusions.* In pre-treated SMZL patients, the association R-B proved to be feasible and induced a high CR rate even in patients with huge splenomegaly. Complete responses were achieved promptly with as low as 4 cycles of therapy but the toxicity observed suggests investigating a dose of Bendamustine lower than 90 mg/m^2. These preliminary results suggest that R-B could represent a potential candidate for replacing splenectomy in the first-line treatment of SMZL patients. Prospective trials aimed to assess toxicity and efficacy of R-B as first line therapy for symptomatic SMZL are warranted.

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SERUM LEVEL OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) AS PROGNOSTIC PARAMETER IN UNTREATED PATIENTS WITH B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. The overall survival of patients with B-cell chronic lymphocytic leukemia (B-CLL) is extremely heterogenous, so novel prognostic factors are being sought in order to identify high-risk patients at the time of diagnosis and to optimize their treatment. Several studies have also shown that in B-CLL there are increased vascularity in bone marrow and increased level of angiogenic cytokines in peripheral blood. *Aim.* In this study, we investigated whether there is an association between serum concentrations of key angiogenic faktor - vascular endothelial growth factor (sVEGF) and mean vascular density (MVD) of bone marrow with conventional prognostic factors (CD38 expression, citogenetic risk, Rai and Binet stage, type of bone marrow infiltration, beta 2 microglobulin) and risk of disease progression in patients with B-CLL. *Material and Methods.* We analyzed 33 Binet untreated stage A patients whose sera were taken at the time of diagnosis and evaluated for the level of sVEGF using ELISA method and whose bone marrow was assessed for MVD using CD34 antibody. *Results.* Serum levels of VEGF positively correlated with Hb level (P=0.027), pletelet number (P=0.007), peripheral blood lymphocytosis (P=0.034) and LDH level (P=0.008). When dealing with MVD only a correlation with CD38 positivity more than 30% (P=0.006) could be found. Different cut-offs of sVEGF level failed to demonstrate any correlation between serum levels of VEGF and time to disease progression. Also, MVD did not correlate with time to disease progression. In univariate and multivariate analyisis the highest prognostic power was obtained when sVEGF and b2-m were examined in combination. Median of progression-free survival of patients who had both sVEGF higher than median value (80pg/mL) and b2-m higher than 3.7mg/mL was only 13 months, while median progression-free survival of patients with one marker increased was 27 months. Patients with both markers below the median experienced the best clinical outcome (median progression-free survival was 48.6 months). *Conclusion.* Only sVEGF in combination with b2-m help us to improve predicts behaviour of disease of stage A patients with B-CLL.

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EFFICACY AND SAFETY OF FLUDARABINE AND CYCLOPHOSPHAMIDE REGIME AS FRONT-LINE THERAPY IN PATIENTS AFFECTED BY CHRONIC LYMPHOCYTIC LEUKEMIA WITH LOW RISK BIOLOGICAL PARAMETERS

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Background. The fludarabine plus cyclophosphamide (FC) regime was reported to be superior to chlorambucil or fludarabine alone in terms of complete response, overall response and progression free survival in previously untreated patients with chronic lymphocytic leukemia (CLL) and since has become standard front-line treatment for this disease. The effect of FCR *vs.* FC in terms of OR rate does not introduce a benefit in patients with mutated IgVH and standard risk FISH, suggesting that biological risk stratification may be necessary to determine the optimal first-line treatment in individual patients. *Aims.* We conducted a multicentre retrospective study to evaluate the efficacy and toxicity of FC administered through the oral and intraveneous route and to assess the influence of immunoglobulin variable region heavy chain (IgVH) gene mutation status, interphase cytogenetic abnormalities, expression of ZAP70 and CD38 on the clinical outcome. *Methods.* We enroled 65 CLL patients treated front-line with oral fludarabine (30 mg/m^2) and oral cyclophosphamide (250 mg/m^2) (38 patients) or intravenous fludarabine (25 mg/m^2) and intravenous cyclophosphamide (250 mg/m^2), administered for three consecutive days every 4 weeks for six

cycles. *Results.* No statistical differences were noticed between the ywo route of administration in terms of overall response (OR), progression free survival (PFS), time to retreatment (TTR) and overall survival (OS). Among the 58 evaluable patients, 31 (53%) achieved a complete response and 18 (31%) a partial response. The median progression-free survival (PFS) was 35 months, median time to re-treatment (TTR) was 42 months and median overall survival (OS) was not reached after 45 months. Haematological toxicity consisted of neutropenia grade III/IV detected in 33 patients and resolved with G-CSF while three patients experienced autoimmune phenomena. Two patients died for infection complications and one due to cardiac death. A significantly lower overall response rate was noticed in the high risk cytogenetic abnormalities group; no statistical differences were detected for the IgVH, ZAP70 and CD38 categories. Response to treatment, high risk cytogenetic abnormalities and unmutated IgVH genes were independent predictors of TTR. Biological parameters were not significant predictors of PFS or OS. *Conclusion.* These results underline the importance of biological stratifications in the front-line treatment of CLL patients. We confirm that the combination of fludarabine and cyclophosphamide is an effective regimen, with mild toxicities that would be especially appropriate in patients with low risk biological parameters who represent in our experience about 30% of patients, in which the association of immunotherapy to FC would not be recommended because of haematological and extra haematological toxicity.

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SPONTANEOUS SECRETION OF SERUM INTERLEUKINS IN PATIENTS WITH B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction. Variation of spontaneous interleukins (IL) production at the malignant diseases of lymphoid system has an important value. Tumor progression at chronic lymphocytic leukemia (CLL) mediately related to the activity of the organism immunocompetent cells. *Aims.* To determine of immunological component influence on a clinical course and therapy response in patients with B-cell CLL. *Methods.* Estimation of interleukins-1β,2,4, tumor necrosis factor α (TNF-α) and interferon-γ (IFN-γ) concentration was conducted for 32 patients with b-cell chronic lymphocytic leukemia (B-CLL) on the different stages of clinical course and treatment. All patients were divided on two groups: group I was composed by 17 patients with B-CLL who had low risk of therapy resistance progress, group II was formed by 15 patients with B-CLL who had high risk of therapy resistance progress. 28 healthy donors represented a control group. *Results.* We revealed the higher concentration of IL-1β in the blood serum in patients with B-CLL in comparison with a control group. It should be noted that in the group II level of IL-1β spontaneous secretion was the highest (38.84±5.9 pg/L). Suppression of IL-2 and IL-4 spontaneous secretion by immunocompetent cells is clearly marked during disease progression. Low level of these cytokines (IL-2 9.63 ±2.3 pg/L, IL-4 1.86±0.3 pg/L) was detected for patients with the high risk of therapy resistant progress. Concentration of TNF-α in the blood serum of the patients with B-CLL correlated with the clinical course and rate therapy resistance. Since TNF-α has the vectored cytotoxic effect on the cells of tumor clone and also immunomodulatory, anti-inflammatory effect, so the increase of this factor quantity testifies to activation of acute phase proteins and possibility of septic complications for patients in the stage of clinical progression. The considerable reduction of IFN-γ concentration in the serum was marked also for patients from both clinical groups, irrespective of clinical course. *Conclusions.* In the patients with B-CLL in stage of clinical progression and high risk of therapy resistance progress high level of IL-1β spontaneous secretion and low level of IL-2, IL-4 and IFN-γ in the blood serum testifies to the unfavorable clinical course. Study of serum interleukines allows to estimate influence of immunological component on the CLL clinical course and also to predict the development of septic complications for patients with CLL.

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PROGNOSTIC FACTORS IN CHRONIC LYMPHATIC LEUKAEMIA. WHAT DO THEY REPORT?

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Introduction. Chronic lymphatic leukaemia (CLL) is one of the most common lymphatic malignancies. The disease is characterised by a highly variable clinical course and prognosis. Newly evaluated biological and cytogenetic markers may allow more precise prediction of the clinical course, however, it is often difficult to judge their impact for the prognosis of the disease from the available literature. The REMARK guidelines developed to improve and guide publication of tumour markers in prognostic studies in a standardized fashion were applied to the currently available CLL literature. *Methods.* We performed a systematic search in MEDLINE, EMBASE and Cochrane CENTRAL with the following inclusion criteria: CLL, at least one new prognostic factor (i.e. IgVH, ZAP70, p53, CD38, del 17p, del 13q, trisomie 12, del 6q or del 11q), one therapy for all patients (or randomised controlled trial), reporting of at least one clinical relevant outcome (e.g. overall survival, response) and a standardised time point of testing the factor (e.g. at diagnosis, at start of treatment). A standardised time point of testing the factor is important because cytogenetic factors may change due to the progress of disease or therapy. We designed a data extraction sheet based on the items of the REMARK guidelines to check the completeness of the eligible publications. *Results.* We screened 5585 abstracts and titles. From 1002 potentially interesting publications, 9 randomised and 5 non-randomised studies were eligible. Most of these articles reported the inclusion criteria of the study and the patient characteristics well (10 of 14 studies). The specimen characteristics and assay methods were not always reported, but 3 of 14 studies gave a complete or partial description of these issues. Survival curves of several factors were shown in 5 of 14 studies. Only 1 of 14 studies reported hazard ratios (HR) at least for one factor. *Summary/Conclusion.* The reporting of new prognostic factors for CLL is often incomplete. Therefore, the reports of these prospective studies do not provide sufficient evidence to judge (e.g. meta-analyse) the impact of new prognostic factors for patients with CLL with respect to overall survival, tumour control or response.

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EFFICACY OF BENDAMUSTINE IN COMBINATION WITH RITUXIMAB IN THE TREATMENT OF ADVANCED STAGE, HEAVILY PRE-TREATED CHRONIC LYMPHOCYTIC LEUKAEMIA

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Background. Bendamustine is a unique chemotherapeutic agent, which resembles anti-metabolites and purine analogues in structure. *in vitro* studies have shown that bendamustine activates DNA-damage stress responses and apoptosis, as well as mitotic catastrophe, a form of necrotic cell death distinct from apoptosis. Bendamustine shows in-vitro synergism with Rituximab, and this combination has been found to be highly effective *in vivo*, with response rates of up to ninety percent in patients with indolent lymphomas. Patients with relapsed and refractory chronic lymphocytic leukaemia have had response rates of 67% with this combination, in a large multi-centre trial. *Aims.* We aim to assess the efficacy and tolerability of Bendamustine and Rituximab in treatment of a small cohort of heavily pre-treated CLL patients, with advanced stage disease, and poor risk cytogenetics in over half the cases. *Methods.* A retrospective analysis was carried out on all patients who received Bendamustine in our institution to date. The medical records of the eligible patients were obtained. Data was gathered regarding age, sex, performance status, stage of CLL, prognostic markers, lines of previous chemotherapy, relapsed *vs.* refractory disease, the number of cycles of Bendamustine and Rituximab received to date, complications of treatment, response rates and duration of follow-up. *Results.* Eight patients were included in the analysis. Five patients have completed treatment, with three patients still receiving treatment. The average age of patients at the start of treatment was 66. All patients had stage C disease. One patient had 17p deletion, three patients had 11q23 deletion and one patient had trisomy 12. Most patients were heavily pre-treated. All had prior exposure to Rituximab, seven to fludarabine, seven to high dose methyprednisolone and two had recieved alemtuzumab. Four patients had relapsed disease, while four patients had refractory disease. Of the eight patients one achieved CR u, four had a partial response, one had stable disease and two had progressive disease, giving an overall response rate of 62.5%. All three non-responding patients had adverse cytogenetics, and had been refractory to previous chemotherapy. The treatment was well tolerated with the most frequent complication being infection, which occurred in five of eight patients, and treatment related cytopaenias in four out of eight patients. Three patients required inpatient admission for treatment of infections. *Conclusions.* In this cohort of heavily pre-treated patients, the overall response rate of 62.5% suggests that Bendamustine and Rituximab can be an effective treatment in relapsed, advanced stage CLL, including those with 11q23 dele-

tion. Treatment was well tolerated in older pateints with marrow failure. We did not find satisfactory response in our patient with a p53 deletion. Although our conclusions are limited by the relatively small numbers of patients available for inclusion in this analysis, our data suggests that larger studies to assess the impact of adverse risk cytogenetics on treatment response are needed.

Table.

Age	Sex	ECOG PS**	No. of Prior Treatments	Cytogenetics	No. of B+R*** cycles to date	Response*	Follow up Period (months)
86	M	1	4	Normal	4	CR (u)	7
64	M	0	8	11q23-	4	PD	2
76	M	1	3	13q14-	6	PR	1
67	M	3	5	Trisomy 12	2	PD	2
48	M	2	7	17p-	2	SD	2
75	F	1	2	11q23-13q14-	4	PR	NA
48	M	0	1	13q14-	1	PR	NA
60	M	0	6	11q23-	1	PR	NA

*Response: (CR (u) = unconfirmed complete remission, PR= partial response, PD = progressive disease, SD = stable disease. **PS= Performance Status. ***B+R= Bendamustine and Rituximab.

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CLINICAL AND IMMUNOLOGICAL CHARACTERISTIC OF ZAP-70POSSITIVE AND ZAP-70 NEGATIVE PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA

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The clinical course of B-cell chronic lymphocytic leukemia is far from being uniform. Some patients have indolent disease with long survival and not require treatment for many years but others have aggressive form of the leukaemia and progress quickly from the initial diagnosis to death within a short time. Several clinical and biological markers each can segregate patients into subgroups with different rates of disease progression. Interest in ZAP-70 has grown since it has been shown, that it is expressed in a subset of cases of chronic lymphocytic leukemia and is associated with aggressive form of disease. We performed analysis of 203 untreated patients with CLL presenting typical morphology and immunophenotype. The mediane age at diagnosis of the patients who participated in our study was 67 years (range 34-86 years). The distribution of patients with Rai stage eg. low-risk (stage 0), intermediate-risk (stage 1 and 2) or high-risk (stage 3 and 4) was 26.6%, 54.19% 19.21% respectively. Patients we segregated by CLL-cell expression of ZAP-70 into two group: ZAP-70 possitive and ZAP-70 negative. Sample analysis included CD38 and ZAP-70 stainings, fluorescence *in situ* hybridization (FISH) for chromosomes 11, 12, 13 and 17, level of bFGF, VEGF and TNF. Patient's samples were obtained after informed consent. In ZAP-70 possitive patients we observed statistically significant increase serum level of TNF, LDH; TTM, percentage of $CD38^+$ cells in peripheral blood and bone marrow and cells with 11q23 deletion. We also found statistically significant short time interval from diagnosis to initial therapy, overall survival, low level of PLT and percentage of $CD4^+$ cells. In all group of patients (ZAP-70 possitive and negative) negative correlation overall survival and time to treatment with percentage of ZAP-70 possitive cells was observed. The percentage of ZAP-70 possitive cell was also statistically significant higher in patients which needed treatment at time of diagnosis. Compare to $ZAP\text{-}70^+/CD38^+$ group, $ZAP\text{-}70^-/CD38^-$ patients showed longer overall survival.

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FLUDARABINE, CYCLOPHOSPHAMIDE AND RITUXIMAB IN FIRST-LINE TREATMENT OF PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA (CLL): RETROSPECTIVE STUDY

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Background. Combination of fludarabine, cyclophosphamide and rituximab (FCR) is currently considered the first-line treatment of choice in physically fit patients (pts) with chronic lymphocytic leukemia (CLL). There is a very limited amount of data regarding the use of FCR in real practice. *Aims.* To perform a retrospective efficacy and safety analysis of FCR regimen used as routine first-line treatment in CLL. *Methods.* We treated 37 pts with active CLL (63% males, median age 58 years [range 37-75]) by FCR regimen as first-line therapy. Diagnosis of CLL, indication for treatment and assessment of response to therapy followed NCI-WG criteria. Patients received standard doses of Fludarabine (25 mg/m^2/i.v. or 40 mg/m^2 p.o d 1-3) and cyclophosphamide (250 mg/m^2 i.v. or p.o. d1-3). Rituximab was administrated i.v. on day 1 of each cycle at the dose of 375 mg/m^2 in all cycles (n=87) or 500 mg/m^2 from 2nd cycle (n=20). Treatment was repeated every 4 weeks. Antimicrobial prophylaxis and growth factors were routinely used. Low/intermediate/high risk according to modified Rai staging was present in 2/62/36%. *Results.* At the time of analysis, the median observation time was 18,3 months (mo).Median number of FCR cycles was 5. The overall response rate/complete response rates were 89/37. Median PFS was 26 mo; median overall survival (OS) was not reached. Grade III/IV neutropenia occurred in 28,17% of cycles and thrombocytopenia grade III/IV in 5,3% of cycles. Serious infections occurred in 3% of cycles. G-CSF was administrated in 54% and recombinant erythropoietin in 23% of pts. *Conclusions.* Treatment of CLL patients in first line with Fludarabine, cyclophosphamide and rituximab resulted in high number of overall and complete responses despite unfavourable prognostic factors present in the majority of pts. Toxicity was acceptable and manageable.

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DASATINIB INDUCES COMPLETE REMISSION OF CHRONIC LYMPHOCYTIC LEUKEMIA IN A PATIENT WITH PRIMARY CNS MANIFESTATION

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Background. Treatment options for central nervous system lymphoma are very limited since the blood-brain barrier prevents free drug penetration and is even less permeable to antibodies. Chronic lymphocytic leukemia (CLL) rarely causes involvement of the central nervous system (CNS). *Case.* We were presented with a case of a 68 year old male who was initially treated for multiple sclerosis without signs of clinical improvement. Further testing revealed a CLL with primary manifestation in the CNS. After initial therapy with MTX/Ifosfamide the patient relapsed and was subsequently treated with high dose Cytosinarabinoside/Mitoxantrone/MTX. Upon disease progression we were able to induce a long lasting complete remission (8 months+) with Dasatinib. *Conclusion.* Among the substances primarily used for treatment of CLL only Cyclophosfamide/Ifosfamide are known to cross the blood brain barrier. Deprived of these options we chose to use Dasatinib as a salvage therapy. Dasatinib is a widely acting tyrosine kinase inhibitor that has recently been demonstrated to be effective in CLL and was shown to pass the blood brain barrier in an animal model. This highly unusual case demonstrates activity of Dasatinib monotherapy in primary CNS-CLL.

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STUDY CONCERNING THE ASSOCIATION BETWEEN CHRONIC LYMPHOCYTIC LEUKEMIA AND DYSLIPIDEMIA

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Background. It is known that the metabolic pathways, namely cholesterol synthesis and adipogenesis are enhanced by CD5 and CD5

expression which is a feature of chronic lymphocytic leukemia. In addition, in previous studies, we have found that there is an association between the metabolic syndrome (which often includes hypertriglyceridemia) and neoplasms. *Aims.* We aimed to study whether the patients with chronic lymphocytic leukemia associate more frequently hypercholesterolemia and hypertriglyceridemia compared to a control group. *Methods.* We have conducted a cross-sectional study that included all patients with chronic lymphocytic leukemia who existed in the records of the Hematology Department of the Emergency County Clinical Hospital from Sibiu in November 2009 and who agreed to participate (group A) and a group of volunteers from the medical staff of the clinics from the same hospital (without neoplasic pathology) (group B). In all subjects a sample from the peripheral blood was taken for determination of hemoleucogram, cholesterol, triglycerides and glucose. The 2 groups were compared and the results were statistically analyzed. *Results.* Group A included 43 patients with a mean age of 69.16±7.74 years; the gender distribution was: 16 women and 27 men. Group B was composed of 34 subjects with the mean age of 32.64±9.88 years; the gender distribution was: 26 women and 8 men. The mean cholesterol level in group A was 203.33±47.76 mg/dL, and in group B - 183.15±40.15 mg/dL (P=0.048); the triglycerides level was in average, in group A 180.73±106 mg/dL and in group B - 81.09±53.33 mg/dL (P<0.00001), the average blood glucose level was in group A 108.12±27.47 mg/dL, and in group B - 92.74±26.36 mg/dL (P=0.015). The cholesterol levels did not correlate with age (P>0.05) or gender (P>0.05), so the difference between the two groups is statistically significant. The triglycerides levels correlated with age (P<0.005) and gender (P=0.016); after adjusting the results by age and gender differences between the two groups remain also statistically significant. Because the glucose level is correlated with age (P<0.05) and gender (P=0.04), the difference between the two groups is not statistically significant. *Conclusions.* Hypertriglyceridemia presented at the patients with chronic lymphocytic leukemia from our study argues for a possible association between the metabolic syndrome and chronic lymphoproliferative syndromes. Hypercholesterolemia presented at the patients with chronic lymphocytic leukemia may have implications in the multiple drug resistance, which is worth studying.

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THE EFFICACY OF FLUDARABINE AND CYCLOPHOSPHAMIDE COMBINATION IN UNTREATED VS PRETREATED PATIENTS WITH B-CELL CHRONIC LYMPHOCYTIC LEUKEMIA

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Background. B-cell chronic lymphocytic leukemia (B-CLL) is a heterogenous disease with an extremely variable course and chemotherapy is ussually not indicated in early and stable disease. Treatment is needed if the progressive form of leukemia started. The combination of fludarabine and cyclophosphamide (FC) is an effective regimen for patients (pts) with B-CLL. *Aim.* The purpose of this study was to analyse the efficacy of fludarabine and cyclophosphamide combination as a first, second and third-line therapy in pts with B-CLL.Treatment outcome was correlated with clinical and biological variables. Matherial and methods. This study included 120 pts with B-CLL who were treated with FC combination between January 2004 and December 2008 in the Institute of Hematology CCS, Belgrade. The diagnosis of CLL was confirmed in all pts using immunophenotyping of pheripheral blood cells. Score 5 (Matutes E) was present in 90% of pts and Score 4 in 10% of pts. Median age was 60 years (range, 30-76) with 78 males and 42 females. 30 pts (25%) were classified as Binet Stage A, 64 pts (53%) as Binet Stage B and 26 pts (22%) as Binet C. Pts received six cycles of fludarabine 25 mg/m^2 i.v x 3 days and cyclophosphamide 250 mg/m^{2}x3 days, every 28 days. FC combination was administrated in 63 pts (52%) as a first line, in 38 pts (32%) as a second- line and in 19 pts (16%) as a third-line therapy. *Results.* The overall response rate (ORR) was 83%. Complete remission (CR) was achieved in 51 pts (42%), very good partial remission in (vGPR) in 15 pts (12%) and partial remission (PR) in 34 pts (28%). The comparasion of response in untreated and pretreated pts shown that CR was achieved in 34 pts (67%) when FC combination was used as first-line therapy, in 11 pts (22%) as a second and in 6 pts (12%) as a third-line therapy. The median duration of remission was 22 months (range, 20-66). The median survival was 52 months (range, 8-120). Fiftyseven pts (58%) who achieved remission had CD 38 expression>30% and shorter overall survival. Lymphocyte doubling time (LyDT) <12 months was present in 57% pts and this was not statistically significant. FC combination caused additional hematological toxicity. Prolonged thrombocytopenia (28% of pts), neutropenia (28% of pts) and anaemia (40% of pts) were observed especially after multiple courses of therapy and in heavily pretreated pts. *Conclusion.* Treatment with fludarabine and cyclophosphamide was associated with a significantly higher CR and ORR in pts with previously untreated CLL and was not associated with increase in episodes of infections.

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THE COMBINATION OF LOW DOSES FLUDARABINE & CYCLOPHOSPHAMIDE AS FRONTLINE TREATMENT FOR ELDERLY PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA.

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Background. Fludarabine (Flu) was the first purine analogue with an oral formulation available for clinical use . Some recent clinical trials, showed that oral-flu was effective and well tolerated, suggesting its convenience in the outpatient setting. It has been previously demonstrated that a combination of reduced doses of flu and cyclophosphamide (Cy), given orally in elderly patients with indolent non Hodgikin lynphoma, showed good efficacy and was simple to administer to patients unfit for more aggressive treatments. *Methods.* Between April 2005 and June 2009, 8 elderly untreated patients (mean age 75, range 68-81) with treatment requiring "B-CLL"(according to ESMO guidelines working group) underwent therapy with low dose of oral fludarabine (25 mg/mq/die) and cyclophosphamide (150 mg/mq/die) both from days 1 to 3 in two dailly administrations. Treatment schedule consisted of 6 cycles repeated at 4 weeks intervals. Patients received antibiotic prophylaxis with trimethoprim/sulphamethoxazole (160/800 mg twice a day, 2 times a week) and allopurinole (300 mg once a day from days 0 to 4) (range 2-6). Performance status was WHO ≤1 in all patients. Comorbidities, which included diabetes, hypertension and chronic heart desease, were present in 7 patients. Biological prognostic factors were assessed at diagnosis and before treatment. ZAP70 and CD38 were independently expressed in 5 and 6 respectively cases. The IgVh sequences were unmutated in 4 patients. Beta2microglobuline mean was 3.2 mg/dL (range 1.6-4.7). The mean of cycles performed was 4 with range 2-6. No patients reduced dose and number of cycle because of haematologic and extra-haematologic toxicities. Specifically 4/8 patients experienced anemia (grade II) but only 2 required epoetin support. Nausea and diarrhea (grade I) were observed in 2 patients. Definition of response was reviewed according to the updated IWCLL-NCI 2008 international general practice criteria. 6 of 8 (OR rate 75%) obtained a response, with 3 molecular complete (CR) and 3 partial response (PR). 3 patients with CR were: CD38$^+$/ZAP70$^+$/IgVH mutated, CD38$^+$/ZAP70$^+$/IgVH unmutated, CD38$^-$/ZAP70$^-$/IgVH mutated. All responder patients are alive and manteined response after mean follow-up of 24 months (range 13-44). *Conclusions.* Based on our studies results, we believe that this regimen could be effective and well tolerated for elderly patients with untreated CLL unfit for more aggressive treatments. In fact although some patients relapsed or progress, most of them do not experience severe toxic side effects or required hospitalisations, obtaining satisfactory quality of life and survival

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EFFICACY OF RITUXIMAB-THERAPY IN RELAPSED OR REFRACTORY HAIRY CELL LEUKEMIA

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Hairy cell leukemia (HCL) is an indolent B-cell neoplasm, strongly expressing the pan B-cell antigen CD20. Alltough high response rates are achieved following 2-chlorodeoxyadenosine (CDA), with longer follow-up relapses are common. We investigated efficacy of Rituximab in 7 patients (pts) (5 males and 2 females) with relapsed or primary refractory HCL after 2-CDA therapy. Rituximab was administered intravenously at a dose of 375 mg/m^2 weekly for 8 weeks. Overall response rate was 75.7%, 3 pts (42.8%) achieved CR, 3 pts (42.8%) achieved a partial response and 1 pts failed to respond. Of the 6 responders followed for a median of 34 months (11-59+) 4 pts (57%) relapsed after 10,17,23 and 36 months. Toxicity was minimal, with no infectious episodes observed. Due to its minimal toxicity and significant efficacy Rituximab is an indication for relapsed or refractory HCL patients after 2-CDA therapy.

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IMATINIB (IMA) PLASMA CONCENTRATION (PC) MONITORING AND ITS CORRELATION WITH TREATMENT RESPONSE IN CHRONIC MYELOGENOUS LEUKEMIA (CML): A SINGLE CENTRE EXPERIENCE

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Background. Correlation between the probability of therapeutic response in patients with CML treated with IMA and IMA PC was recently described, suggesting that IMA trough PC levels should be above 1.04 ng/mL. However data from large group of "real-life" CML patients (out of clinical trials), where IMA PC monitoring is performed, are still lacking. *Aims.* To evaluate correlation of IMA trough PC with daily IMA dose, treatment response and influence of alfa1-acid glycoprotein (AGP) on IMA PC in "real-life" group of CML pts. *Methods.* HPLC was used for IMA trough PC monitoring. Pts. charts were assessed for evaluation of treatment response and AGP concentration results. *Results.* Between 9/2008 and 1/2010 IMA PC have been detected in 397 samples from 108 pts. (median: 3 samples/ pt., range: 1-22). Median IMA daily dose at the time of monitoring was 400 mg (range: 150-800 mg). Median duration of IMA treatment at the time of sampling was 28 months (range: 0.5-99). Median IMA PC for pts. with 24 h. IMA dosing was 830 ng/mL (n=83 pts.) and for 12 h. dosing 1450 ng/mL (n=14 pts.). Mean inter-patient and intra-patient variability of IMA PC was 31% and 17%. There was a clear correlation between daily dose of IMA and IMA PC (Figure). IMA PC below suggested level 1.0 ng/mL was measured in 57% of samples and this percentage decreased with increasing daily dose of IMA (65% in 300 mg/day group, 60% in 400 mg/day, 10% in 600 mg/day and 0% in 800 mg/day group). 67 pts. with IMA as first line therapy were further analyzed regarding correlation of their IMA PC and achieved cytogenetic response. Medians of all samples obtained from single pt. were used for analysis. Any significant difference was found between medians of IMA PC in pts. with and without CCyR - 0.938 ng/mL *vs.* 0.836 (P=0.560, Mann-Whitney test). Finally our analysis found statistically significant correlation between IMA PC and AGP plasma concentration at the time of sampling - for different ranges of AGP concentrations the medians of IMA PC were as follows: AGP < 0.5 mg/mL - IMA PC 0.780 ng/mL, AGP 0.5 to 1.0 mg/mL - IMA PC 0.940 ng/mL, AGP 1.0 to 1.5 mg/mL - IMA PC 1.10 ng/mL, AGP > 1.5 mg/mL - IMA PC 1.24 ng/mL (P<0.001) (Kruskal-Wallis ANOVA). *Summary/Conclusions.* Our analysis did not find any correlation between IMA PC and cytogenetic response in patients with CML treated with IMA as first line therapy. On the contrary, our analysis showed that the resulting PC IMA strongly depends on the current administered dose of IMA and the current AGP concentrations at the time of sampling.

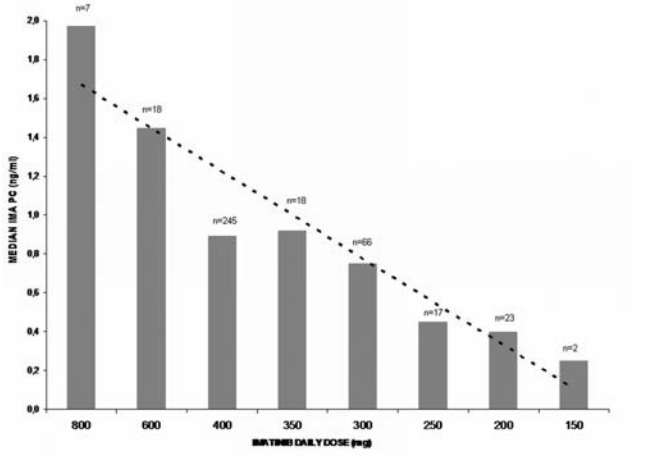

Figure. Correlation of IMA PC and daily dose of IMA.

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A NOVEL MECHANISM OF NILOTINIB-INDUCED APOPTOSIS; BIOACTIVE SPHINGOLIPIDS

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Background. Chronic myeloid leukemia (CML) is a hematological malignancy resulting from the reciprocal translocation of chromosomes 9 and 22 that generates BCR/ABL oncogene. Nilotinib is a rationally designed, specific BCR/ABL tyrosine kinase inhibitor. Ceramide is a novel regulator of cell growth and proliferation, differentiation, senescence, cell cycle, and also acts as a strong apoptotic molecule while its conversion to antiapoptotoic glucosyleceramide (GC) and sphingosine-1-phosphate (S1P) by glucosyle ceramde synthase (GCS) and sphingosine kinase-1 (SK-1) enzymes result in more aggressive and resistant cancers. *Aim.* In this study, we examined the roles of ceramide generating and ceramide clearence genes in nilotinib induced apoptosis and possibility of increasing the sensitivity of BCR/ABL positive K562 and Meg-01 cells to nilotinib through targeting ceramide metabolism. *Methods.* Expression levels of ceramide synthase (LASS1-6) genes, SK-1, and GCS genes in response to increasing concentrations of nilotinib in K562 and Meg-01 cells were examined by RT-PCR. The antiproliferative effects of nilotinib, ceramide analog, GCS and SK-1 inhibitors were conducted by XTT cell proliferation assay. The changes in caspase-3 enzyme activity and loss of mitochondrial membrane potential (MMP) were measured by caspase-3 colorimetric assay and JC-1 MMP detection kit, respectively. *Results.* Importantly, RT-PCR results demonstrated that there were significant decreases in expression levels of SK-1 and increases in expression levels of Lass1, Lass2, Lass4, Lass5, and Lass6 ceramide synthase genes in response to nilotinib in a dose dependent manner as compared to untreated controls in both K562 and Meg-01 cells. On the other hand, application of ceramide analogs/mimetics and inhibitors of GCS and SK-1 enzymes inhibited cell proliferation and induced apoptosis in a dose dependent manner. Our results also revealed that combination of even very low doses of nilotinib with these bioactive sphingolipids targeting chemicals resulted in synergistic apoptosis in both cells, as compared to any agent alone. The highest apoptotic rates were observed in combination of nilotinib with ceramide analog, C8:ceramide. *Summary/Conclusion.* It was shown for the first time by this study that nilotinib triggers apoptosis by regulating intracellular concentrations of bioactive sphingolipids in addition to inhibition of BCR/ABL in K562 and Meg-01 CML cells. Increasing endogenous ceramide in addition to inhibition of BCR/ABL could be an effective treatment method to regulate CML cell growth.

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NILOTINIB SYNERGIZES WITH DASATINIB IN PRODUCING GROWTH-INHIBITION IN CML CELLS, INCLUDING BCR/ABL T315I+ CELLS

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In most patients with chronic myeloid leukemia (CML), complete cytogenetic remission can be achieved with the BCR/ABL tyrosine kinase inhibitor (TKI) imatinib. However, unfortunately, not all CML patients are long-term responders. Acquired resistance against imatinib is often caused by BCR/ABL mutations. In most of these patients, a second generation TKI is prescribed. However, the T315I mutant of BCR/ABL introduces resistance against most TKI, including nilotinib and dasatinib. One approach to overcome drug resistance in BCR/ABL T315I$^+$ CML cells may be to apply drug combinations. Recent data suggest that the mechanisms through which dasatinib and nilotinib act on BCR/ABL differ from each other and that both drugs may elicit mild residual activity on most BCR/ABL mutants including T315I. Moreover, it has been described that both drugs act on multiple additional targets in CML cells. We here show that dasatinib and nilotinib cooperate with each other in producing growth inhibition in BCR/ABL T315I$^+$ CML cells. Strong cooperative or even synergistic effects were observed in primary T315I$^+$ CML cells in 3 of 3 patients tested (chronic phase, n=1; blast phase n=2) as well as in Ba/F3 cells exhibiting BCR/ABL T315I. Synergism was demonstrable at pharmacologic drug concentrations (<1 μM) and was also seen in freshly diagnosed patients

with CML (wt BCR/ABL+ leukemic cells) and in all other BCR/ABL mutants tested, suggesting that the drug combination acts suppressive on most if not all CML subclones. Synergistic effects of dasatinib and nilotinib may be explained by direct effects on BCR/ABL including residual effects on BCR/ABL T315I, and additional drug targets in CML cells. Notably, the target kinase spectrum for Nilotinib in primary CML cells was found to differ significantly from the target kinase spectrum of Dasatinib. Together, our data show that two second generation BCR/ABL TKI, Dasatinib and Nilotinib, produce synergistic drug effects on TKI-resistant CML cells including leukemic cells exhibiting BCR/ABL T315I. These drug-combination effects occur at drug concentrations that can be reached *in vivo* and may thus represent an interesting new pharmacologic concept to treat patients with TKI-resistant CML.

1303

CORRELATION BETWEEN PRAME, WT1 AND BCR-ABL EXPRESSION AND RESPONSE TO THE THERAPY AT CML

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Background. It was shown that WT1 and Prame genes are expressed at CML. The data about correlation between WT1 and BCR-ABL expression and the role of WT1 in relapse prediction are contradictory. It is suggested that Prame expression increases during disease progression of CML, correlation was shown between Prame expression and response to nilotinib therapy. The question of interaction between Prame, WT1 and BCR-ABL during disease development and degree of significance WT1 and Prame as markers of response to the therapy remains open. Aim. To analyze Prame, WT1 and BCR-ABL expression, the correlation of expression with the response to the therapy CML patients at different timepoints. *Methods.* 92 patients were included: 26 primary patients, 36 at the point of 12 month and 30 at the point of 18 month of imatinib therapy. RQ-PCR testing was used for genes expression definition. The patients were divided into 4 group depending of the response to the therapy during the next 12-24 month from this timepoint: 1) resistant/bad response; 2) secondary resistant; 3) suboptimal response; 4) good response. In each timepoint the patients were divided also into 2 groups: patients having achieved major molecular response (MMR) on imatinib therapy and not achieved. Spearman's rank correlation and Pearson's chi-square test for contingency tables was used for statistical analysis. *Results.*There was not significant dependence between response to the therapy and Prame, WT1 and BCR-ABL expression, and dependence of Prame and WT1 expression with BCR-ABL expression in primary patients, but there was strict correlation between Prame and WT1 expression (Rs_corr=0,44, P<0,05). At the point 12 month of therapy there was significant dependence between BCR-ABL and Prame expression (Rs_corr=0,36, P<0,05), BCR-ABL and WT1 expression (Rs_corr=0,52, P<0,001), between BCR-ABL expression and response (Rs_corr=0,84, P<0,001), Prame expression and response (Rs_corr=0,34, P<0,05), WT1 expression and response (Rs_corr=0,54, P<0,001). The correlation between level of WT1 expression and achievement of MMR was also significant (P<0,05), such correlation was not proved for Prame expression. At the point 18 month of therapy there was significant dependence between BCR-ABL and WT1 expression (Rs_corr=0,66, P<0,001), between BCR-ABL expression and response (Rs_corr=0,78, P<0,001), WT1 expression and response (Rs_corr=0,39, P<0,05), correlation between level of WT1 expression and achievement of MMR (P<0,05). Significant correlation was not found between BCR-ABL and Prame expression, Prame expression and response, Prame expression and achievement of MMR. *Conclusion.*The dependence between expression of Prame, WT1, BCR-ABL and between expression of these genes and response to the therapy is changing during CML development and treatment. Correlation between BCR-ABL and WT1 expression, WT1 expression and response to the therapy is more strict and renewed than the same markers for Prame.

1304

DOWNREGULATION OF ANTIAPOPTOTIC GENES INDUCED BY IMATINIB/MELPHALAN COMBINATION INCREASES KILLING OF CHRONIC MYELOID LEUKEMIA CELLS

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Imatinib (IM) has become the drug of choice for first line therapy in the treatment of chronic myeloid leukemia (CML). However, a relatively small percentage of CML patients develop resistance to IM and there is a need for alternative strategies. In this perspective, we have already demonstrated that a sequential exposure of K562 cells to IM followed by Melphalan (MEL), induces a greater cell death than each drug alone. This synergistic activity was also confirmed in primary cells from bone marrow of 6 CML patients at diagnosis. The IM/MEL combination was the most effective treatment in reducing the total number of CFU-GM and BFU-E colonies (P<0.05 *vs.* IM alone) and especially the number of BCR/ABL copies (P<0.01 *vs.* IM). (Giallongo C. *et al.,* submitted). In order to evaluate the possible mechanism of action and the superior efficacy of the combination, we analyzed several apoptotic pathways on K562 cells by TaqMan Low Density Arrays. Treatment with MEL alone induced expression of FASL, FAS and FADD (respectively of 1000, 400 and 40 fold), that leads to caspase-8 activation. APAF and PYCARD (20 and 75 fold) observed over-expression could also activate caspase-9. Furthermore, MEL-induced activation of mitochondrial apoptotic pathway by BAD, BAK1 and BAX (7×10^6, 40 and 40 fold) was counterbalanced by BCL2, BCL-xL and BIRC8 (80, 12 and 50 fold) expression. In addition, MEL activated BIRC1 (1500 fold), BIRC3 (3000 fold) and, especially, BIRC7 (>109 fold). MEL alone also induces over-expression of genes involved in NFkB activity such as NFKB1, NFKB2, IKBKE and RELB (7, 50, 150 and 800 fold). In the TNF superfamily system MEL both up-regulated an apoptotic pathway, TRAIL (1600 fold) and its receptors DR4 and DR5 (30 and 25 fold) with consequent activation of caspase-8, and an anti-apoptotic one, TNFβ gene (800 fold) and its receptor TNFR2 (20 fold), leading to activation of NFKB. IM alone is able to down-regulate all above mentioned anti-apoptotic genes. Unexpectedly, IM/MEL combination did not dramatically increase expression of apoptotic genes, rather, the association silenced the anti-apoptotic signaling induced by MEL alone. In conclusion, the greater cytotoxic effect observed with IM/MEL combination is probably due to a shift towards the pro-apoptotic gene expression profile. In fact, MEL alone induces both pro- and anti-apoptotic genes while IM alone is able to down-regulate anti-apoptotic pathways. IM/MEL association keeps the ability to cause apoptosis and switches off the anti-apoptotic defensive signal induced by MEL alone.

1305

THE RATE OF BCR-ABL KINASE DOMAIN MUTATIONS IN CML PATIENTS RESISTANT TO TYROSINE KINASE INHIBITOR TREATMENT IN SOUTH AFRICA, UNUSUAL FINDINGS

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Background. Imatinib Mesylate, the standard first line tyrosine kinase inhibitor treatment, has revolutionized the management of chronic myeloid leukemia (CML) patients. Nonetheless a minority of patients either fail to respond or develop resistance to treatment. BCR-ABL kinase domain and p-loop mutations are the best understood cause of resistance. *Aims.* The aim of this study was to ascertain the frequencies and types of mutations occurring in South African CML patients treated with Imatinib. *Methods.* The levels of BCR/ABL were monitored via quantitative real time PCR (RQ-PCR) in CML patients receiving TKI therapy from nine centers across South Africa. The BCR-ABL gene was sequenced in 147 patients, on request of the treating physician. A semi-nested reverse transcription polymerase chain reaction (RT-PCR) was performed to selectively amplify exons 4-7 of the ABL kinase domain that was subsequently sequenced using direct sequencing. To analyze these results, patients were divided into three groups based on their response to TKI treatment. First, responders who became resistant to treatment (increased

levels of BCR-ABL above the 3 log level in two consecutive assays), Second patients who failed to respond and had primary resistance (failure to obtain a CCR at 18 months) and third patients for whom we did not have further information on their response to therapy. *Results.* Among forty patients with acquired resistance, 42.9% showed a BCR-ABL gene mutation. The most common mutations were M244V (17.4%), E255K (13%) and Y253H (13%), with nine different mutations accounting for the remaining 56.6%. Forty one patients failed to respond to therapy, and the mutation rate in this group was 29.3% with the T315I (28.6%) being the most frequently observed. No detailed information was available for 63 TKI resistant CML patients. In this group, the mutation rate was 28.6% with the T315I (20%) and E255K (20%) being the most frequent mutations, followed by M244V (15%). Overall, among the 147 patients, the mutation rate was 36.77%. The T315I (17.5%), M244V (12.3%) and E255K (12.3) were the most frequently detected mutations, with 22 other mutations being detected. *Summary/Conclusions.* While the mutation rate in patients who acquired resistance to Imatinib was comparable to those observed in other studies, mutations in the BCR-ABL gene only accounted for 29% of patients with primary resistance. This finding warrant further investigations as to other reasons for primary resistance which are likely to be multi-factorial. Overall, the most frequent mutation was the T315I (17.5%). Our results have set a base line for the types of mutations which may be expected in South African CML patients. Fifty two percent of the mutations detected resulted in full resistance to TKI therapy, which poses a large problem for the future management of patients in an already over burdened public health system.

1306

COMPARATIVE ANALYSIS OF DIFFERENTIAL GENES EXPRESSION OF CHRONIC MYELOID LEUKEMIA BETWEEN CHRONIC AND BLAST PHASE WITH CDNA MICROARRAY

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Background. Chronic myeloid leukemia (CML) is characterized by the clonal expansion of hematopoietic stem cells (HSCs). The natural history of CML progresses from a relatively benign chronic phase into a fatal blast crisis, which is incurable by chemotherapy. Despite recent success in the treatment of early-stage disease (chronic phase, CP), blastic phase (BP) of chronic myeloid leukemia remains a therapeutic challenge. The better understanding of the molecular mechanisms underlying disease progression constitutes the most important problem in predicting and detecting patients involved into advanced disease and in alternating therapeutic strategies. *Aims.* The aim of this study was to explore the mechanism underlying the acute transformation of CML by comparing the differential genes expression in CP and BP. *Methods.* Analysis of gene expression profiles of 4 pairs of Chinese chronic myeloid leukemia patients (4 CP patients *vs.* 4 BP patients, Appropriate consent for bone marrow samples from each individual was obtained) was carried out by using cDNA microarray represented with 4096 genes. *Results.* 74 differential expression genes were identified in at least 3 pairs of CML patients, in which 52 genes were down-regulated and 22 genes were up -regulated in BP patients. Only 5 genes (FCN1_TPX1_ANXA3_BTG2_HCLS1) which down-regulated in BP were identified consistently in all 4 pairs of patients. The expression of selected genes was validated by semi-quantitative RT-PCR. The functions of the differential genes were involved in cell structure/mobility, signal transduction, gene transcription, related immunity, metabolism, cell cycle, protein translation/synthesis and other unknown functions. *Conclusions.* Our results showed that significant difference of gene expression profile existed between chronic and blastic phases of CML. The different genes were concerned with cell signal transduction cell cycle regulation, cell differentiation and related immunity, and might be the critical genes for chronic myeloid leukemia blast crisis transformation.

1307

TRIPLE POINT MUTATIONS, WITH TWO WITHIN THE SAME CODON IN A CML PATIENT RESISTANT TO TYROSINE KINASE INHIBITOR THERAPY

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Background. In chronic myeloid leukemia (CML) patients, resistance to the tyrosine kinase inhibitor (TKI), Imatinib, is often due to point mutations in the ABL kinase domain of the BCR-ABL gene. From studies performed to date, only a small number of patients with multiple point mutations have been identified. As the position and nature of these mutations can influence drug resistance, revised patient-specific treatment strategies, including combination TKI therapy may prove beneficial in treating patients with multiple mutations. We report on the occurrence of multiple mutations in Imatinib-resistant CML patients and describe findings on a patient with three mutations in the ABL kinase domain. Although previous studies have detected three or more mutations, this is the first study to report on two mutations occurring at different nucleotides within the same codon, in addition to another third mutation. *Aims.* To determine the frequency of multiple point mutations in the ABL kinase domain in a cohort of South African Imatinib-resistant CML patients and to characterize the type and location of mutations in a patient with a triple mutation. This will emphasize the need to optimize treatment strategies in patients with multiple mutations. *Methods.* Samples from 155 Imatinib-resistant CML patients were analyzed by direct sequencing of the ABL kinase domain. Resistance was suspected on the basis of two consecutive increased levels of BCR-ABL using real-time quantitative polymerase chain reaction results or failure to obtain a major cytogenetic response within 18 months. In the patient harboring the triple mutation, samples were analyzed at 40, 46, 49 and 55 months to determine the levels of BCR-ABL and to detect any kinase domain mutations. Drug efficacy for each mutation was determined from reported data. *Results.* Of the patients sequenced, 56 (36.1%) had detectable point mutations at 25 different residues. Seven of these 56 patients (12.5%) had double mutations and one patient (1.8 %) had three mutations. Despite dose escalation, the triple mutation patient showed no response to Imatinib. Mutational analysis forty months after diagnosis showed that two of these mutations (G to A and A to T) occurred at adjacent nucleotide positions within codon 255. This could result in the substitution of Glutamic acid with either a Methionine, Valine or Lysine. The third mutation substituted a Glutamine with a Histidine at codon 252 (Q252H). These three mutations were all previously reported to be fully resistant to Imatinib and partially sensitive to Dasatinib and Nilotinib. The patient then rapidly transformed in blastic phase, was given Nilotinib, but after a poor response this was withdrawn. However, sequencing analysis after Nilotinib therapy showed that although the Q252H mutation was undetectable, the two mutations in codon 255 still remained, suggesting that the Q252H mutation was more sensitive to treatment. *Summary/Conclusions.* The findings of this study highlight the importance of testing for kinase domain mutations in therapy resistant CML patients and that multiple mutations, although rare, can occur. It also emphasizes the value of patient-specific optimized treatment strategies dependant on the location and nature of any mutations present.

1308

BCR-ABL SEQUENCING AND MUTATIONAL STATUS IN CHRONIC MYELOID LEUKEMIA: DESCRIPTION OF ATYPICAL MUTATIONS AND RELATIONSHIP WITH RESPONSE TO IMATINIB AS A PRELIMINARY ANALYSIS OF A SINGLE CENTER STUDY

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Background. The clinical significance of mutations of bcr-abl at diagnosis of chronic myeloid leukemia and during its clinical course is not well established except in well-studied mutations and unresponsive to tyrosine kinase inhibitors such as T315I, being controversial the utility of mutational analysis for therapeutic decisions. We present preliminary results of a study of mutation by directing sequencing analysis in CML patients with alterations in the detection of exons 7, 8 and 9. *Aims.* To analyze the mutational status by sequencing a group of CML patients by establishing correlation with response to treatment with imatinib. *Methods.* In January of 2009 it began in the Hematology Department of the Hospital Virgen del Rocío de Sevilla a study of bcr-abl mutations in CML patients at diagnosis and, in retrospect, in those treated with imatinib who presented still detect bcr-abl transcripts using real-time PCR. All patients were treated with imatinib after diagnostic doses of 400 mg daily, with subsequent monitoring as recommended by the European Network of Leukemia (2006). The mutational study was performed by extracting RNA from peripheral blood samples, reverse transcription to cDNA, gene amplification by nested PCR and subsequent direct sequencing. We conducted a subanalysis of the first 10 patients included correlating the results with response to imatinib. *Results.* Four out of each studied patients submitted mutations. Three of them consisted of a deletion of exon 7 and one in an insertion between exons 8 and 9. These two types of mutations may potential-

ly alter the conformation of the kinase domain of BCR-ABL protein. Three of the mutated patients (75%) had a suboptimal response to treatment with imatinib, without greater molecular response achieved after 18 months of follow up, leading to an increase in dose or change to second inhibitor (nilotinib or dasatinib) without intolerance. One patient with deletion of exon 7 shows a complete hematologic and cytogenetic response transcripts persisted over follow-up of 0.1% with less than 18 months. *Conclusions.* 1) Mutations in the bcr-abl kinase domain of exon 7 deletion type and insertion between exons 8 and 9, rarely reported in literature, determined in our series poorer response to treatment with imatinib in a 75% CML patients. The value of its presence at the time of diagnosis must be confirmed after further recruitment and monitoring of patients in the study. 2) The use in our study of the direct sequencing method in the analysis to detect mutations more rarely described and that might go unnoticed by other methods.

1309

PROGNOSTIC SIGNIFICANCE OF CHROMOSOMAL REARRANGEMENTS IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA TREATED WITH IMATINIB

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Background. The Philadelphia chromosome (Ph1) is the hallmark of chronic myeloid leukemia (CML) and results from the reciprocal translocation t(9;22)(q34;q11). Glivec (Imatinib mesylate) is included on the orphan drugs list, works on a molecular level. CML treatment is guided by monitoring cytogenetic and molecular therapy response. Complex chromosomal rearrangements developing during progression of CML are rather rare and significance and frequency of different anomalies are poorly understood. *Method.* The study was conducted between January 2002 and January 2010. The study group included 35 patients: 19 males and 16 females. The average age was 38,11 years. All the patients received a daily dose of 400 mg Imatinib. The cytogenetic studies were made on hematopoietic bone marrow using culture for 24-48 hours on a culture medium dedicated for hematopoietic cells, followed by standard cytogenetic exam, GTG banding and karyotyping. The haematologic and cytogentic evaluation of patients were monitored during the treatment. The patients were classified on the basis of the response to treatment (% of Ph^+ cells) in 5 categories: complete cytogenetic response (0% Ph^+ cells), major cytogenetic response (<35% Ph^+ cells), partial response (1% to 34% Ph^+ cells), minor cytogenetic response (35% to 90% Ph^+ cells) and without cytogenetic response.

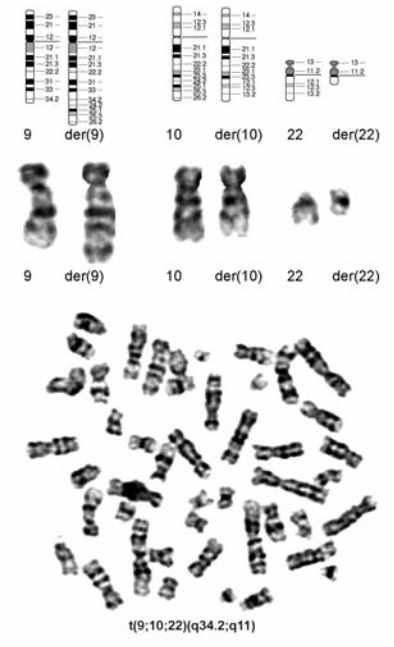

Figure.

Results. The average time for the treatment was 20,8 months with limits between 4 and 65 months. At 6 months of treatment 85,71% obtained CCR and 14,29% mCR. At the last evaluation, CCR was obtained by 67,85% patients, MCR by 10,71% and mCR by 21,42%. Several patients acquired additional clonal cytogenetic abnormalities during therapy, a finding with significant implications for prognosis and laboratory monitoring. The majority of structural changes were unbalanced. Variant Ph translocation (involving chromosome 9, 22 and one or more other chromosome) were found in 3 patients, the rest of the cohort had a classical Ph translocation associated with additional structural aberrations. *Conclusions.* Glivec is a revolutionary drug which grew the life expectance of patients with CML. Complex chromosomal rearrangements are associated with rather poor prognosis and respond poorly to antileukemic treatment. Identification of complex chromosomal aberrations may provide information of genetic mechanisms playng role in disease progression.

1310

MOLECULAR MONITORING OF CHRONIC MYELOCYTIC LEUKAEMIA (CML) IN A PATIENT POPULATION OF THE STATE OF QATAR ON IMATINIB MESYLATE (IM) TREATMENT

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Background. Most cases of CML are associated with presence of BCR-ABL fusion gene; this molecular anomaly enabled the development of the drug Imatinib that selectively target diseased cells. The success of the rationally designed Imatinib therapy is tempered, however, by the problem of disease persistence and resistance. Internationally, around 10% of CML patients develop resistance to Imatinib. In comparison the rate of resistance in Qatar was noticed to be higher; as 40-50% of patient's progress from chronic phase to accelerated or blastic crisis phase within the 1st year of treatment. *Aim.* To monitor the molecular response of CML patients in the state of Qatar using Real Time PCR (RQ-PCR) in order to provide an early indication of emerging drug resistance. *Materials.* CML PB and BM samples were analysed using RQ-PCR as per the European against Cancer (EAC) protocol. *Results.* Over a period of three years, a total of 113 samples (61 PB and 52 BM) from 24 CML patients receiving Imatinib underwent molecular analysis, via serial real-time-polymerase chain reaction (RQ-PCR) to monitor the ratio of BCR-ABL to normal ABL transcripts. According to European Leukemia Net (ELN) a BCR-ABL/ABL ratio of 0.1% or less represents major molecular response (MMR), BCR-ABL/ABL ratio of (0.1% to 1%) represents minor molecular response (MiMR), BCR-ABL/ABL ratio of more than 1% suggested a resistant or relapsed case. As recommended by ELN, major molecular response (MMR) has to be achieved at 18 months after diagnosis. The rate of resistance in our patients was found to be as high as 45%.8/24 patients (33%) showed resistance within 6-39 months (mean & median = 13 months).3 patients (12%) developed additional chromosomal abnormalities (ACA) +8, +14, +17, +19. One patient of the three developed AML at 21 months and died, the other developed ALL and died at 9 months, and the third kept showing fluctuating results. On the other hand, 2 patients (9%) showed an initial response after 6 months (mean & median = 18 months) one still in remission and the other in (MiMR), 1 patient (4%) showed initial response after 15 month and still in remission, 2 (9%) patients achieved MMR at 18 and 21 months. 8 patients (33%) enrolled recently into the study and are still too early to yield any significant results at the time of writing this abstract. *Summary/Conclusion.* Our results confirmed the higher resistance rate of Qatari patient's population in comparison to European and international counterparts. 45% Qatar: 10-15% Europeans. We noticed that patients responding early to treatment had a good chance to achieve MMR and disease-free survival (DFS); while patients not achieving any molecular response during the 1st year were more likely resist to Imatinib treatment. Although the recommendation of ELN is to monitor molecular response at 12 months of treatment (not earlier), however, in areas with high resistance rate other monitoring guidelines are needed. We thus suggest incorporating molecular response at ELN criteria as an essential early monitoring test at 6, 9 months to allow early prediction of resisting / relapsing patients.

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THE CLINICAL IMPACT OF THE IMATINIB BLOOD LEVEL TESTING FOR CML: SINGLE CENTER EXPERIENCE OF FUNDENI HEMATOLOGY DEPARTMENT IN COLLABORATION WITH BORDEAUX'S PHARMACOLOGY AND TOXICOLOGY LABORATORY

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The patients with chronic myeloid leukemia (CML) in chronic phase who receive Imatinib from diagnosis have a very good prognosis, but, despite of this, cases of treatment failure or suboptimal response have been reported. Achieving maximum benefit with Imatinib therapy may require optimal dosing as well as adherence to therapy. To look for these, Imatinib Blood Level Testing (BLT) had been done for some of our patients with CML. In the last three years, we sampled 40 probes which were send to Department of Clinical Pharmacology and Toxicology, Centre Hospitalier Universitaire (CHU) de Bordeaux. According to ELN recommendations, the indications for Imatinib BLT were: insufficient response for thirty two patients (80%), suspicion of non-adherence to treatment for five patients (12,5%) and adverse events for three patients (7,5%). The median time from the beginning of Imatinib treatment of those patients is 65,7 months (raging from 12-96 months). In the moment of sampling, the molecular monitoring showed that six patients (15%) were in complete remission and thirty four patients (85%) were in partial remission and progresive disease. Compared to the medium value, recommended by Bordeaux lab (1002 ng/mL), the results of Imatinib BLT were high in twenty five patients (62,5%), low in twelve patients (30%) and normal in three patients (7,5%). After the results had been received, twenty patients (50%) were tested for mutations in BCR-ABL- kinase domain. For nineteen patients (47,5%) the Imatinib dose was escalated, and one patient (2,5%) had been retested after confessing non-adherence to therapy. In CML patients with suboptimal response to Imatinib at 400 mg per day, monitoring blood levels of Imatinib would be most helpful to physicians in determining if the dose should be increased, or whether, in the case of imatinib failure, alternative therapies should be considered.

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DETECTION OF ABL KINASE DOMAIN POINT MUTATIONS IN 11 CHRONIC MYELOID LEUKEMIA PATIENTS RESISTANT TO IMATINIB TREATMENT

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The Philadelphia (Ph1) chromosome which characterizes the chronic myeloid leukemia (CML) is generated by a balanced reciprocal translocation t(9;22)(q34;q11), in which the ABL and BCR genes are disrupted, to form a BCR-ABL fusion gene. Most CML patients express the BCR-ABL transcript with β2α2 or β3α2 junction. A few CML patients express a shortened BCR-ABL transcript with an e1a2 junction (m-bcr) and only sporadic cases have a BCR breakpoint located in the μ-bcr region and express the rare junction e19a2. The recent introduction of tyrosine kinase inhibitors such as imatinib represents a major therapeutic advance in the management of CML. However, resistance to imatinib due to point mutations in Bcr-Abl kinase domain is an emerging problem. This study was purposed to evaluate ABL tyrosine kinase point mutations in 11 chronic myeloid leukemia (CML) patients showing resistance to imatinib. All the patients disclosed the Ph1 chromosome revealed by R-banding karyotype. Multiplexed reverse transcription polymerase chain reaction (RT-PCR) was performed to discover the BCR/ABL subtype. We have identified 6 patients with β3α2 rearrangement, 4 patients with β2α2 rearrangements and one patient carried the e19a2 transcript. A cytogenetic follow-up during imatinib treatment was done and none of the 11 patients achieved complete cytogenetic response throughout a mean duration of 21 months. A search for a mutation on ABL gene exons 4-9 was done by sequencing cDNA for the BCR-ABL fusion transcript. The results showed that the point mutations were found in 2 of 11 patients. One patient showed E355G mutation. This one was detected in the majority of alleles. It has been recognized that E355G is a point mutation in the exon 6 of BCR-ABL gene that alters the conformation of the kinase domain required for imatinib binding and therefore, decreased sensitivity to imatinib. This is, to our knowledge, the first report of CML with e19a2 transcript and E355G mutation arising during imatinib treatment. In the second patient, two point mutations have been identified: L376S and K467R which probably cause resistance to imatinib, the mechanisms by which resistance is induced, are discussed. These findings indicate that E355G induces imatinib resistance also in CML patients carrying the e19a2 transcript. We speculate that DNA instability in this type of CML may not differ significantly from that in the M-BCR-ABL type, and that blastic transformation with clonal evolution eventually occurs unless suitable therapy is administrated. We have identified two point mutations that have not been described previously in the literature, and induce resistance to imatinib therapy. It is concluded that ABL kinase point mutation is an important mechanism of imatinib resistance, monitoring the ABL kinase domain point mutation is helpful to estimating the prognosis and adjusting the therapeutic strategy.

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ABSENCE OF BCR-ABL KINASE DOMAIN MUTATIONS IN CHRONIC MYELOCYTIC LEUKEMIA (CML) QATARI PATIENTS ON IMATINIB MESYLATE (IM) TREATMENT

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Background. More than 45% of CML patients in Qatar resist the first line of Imatinib treatment; we thus investigated the role ABL mutations might play in this resistance. *Aim.* To investigate the role of ABL mutation in resistance of CML patients to treatment in Qatar. *Materials & Methods.* CML PB and BM samples were collected from 21 patients; total RNA was extracted using QIAamp RNA Blood Mini Kit, cDNA synthesis was performed using RT-Dx, cDNA was amplified using Semi-nested PCR, the 863 bp PCR product was directly sequenced in the forward and reverse directions using ABI PRISMA 3130 Genetic analyzer. *Results.* Over a period of three years, 57 samples (33 PB and 24 BM) for 21 patients receiving Imatinib were studied for ABL mutations prior to treatment, and at the time of resistance (failure and suboptimal response as defined by European Leukaemia Net (ELN)). Initially, sequencing was performed on 412 bp of ABL gene alone using ABL forward and reverse primers, the results didn't show any mutations in ABL kinase domain at C helix, SH3 contact, IM binding site, SH2 contact, kinase catalytic domain and Activation loop. In order to avoid wild type ABL amplifications and cover the whole ABL kinase domain that is fused to BCR, we resequenced the 57 samples using two new sets of primers that amplified BCR-ABL fusion gene followed by semi-nested PCR that amplified the whole ABL kinase domain (863 bp). We could find no evidence of ABL gene mutations at P-loop, C helix, SH3 contact, IM binding site, SH2 contact, kinase catalytic domain, Activation loop, C-terminal loop. To confirm the absence of any novel mutation we compared the resulted sequence data with ABL kinase domain reference sequence (GenBank accession no. M14752) using www.ncbi.nlm.nih.gov/blast. *Summary/Conclusion.* Due to high rate of resistance of Qatari CML patients to Imatinib, we tested our patients for BCR-ABL mutation. Internationally, ABL mutations are the most common cause of Imatinib resistance. Despite of the high resistance in our patient's population, our study didn't reveal any of the known imatinib resistant ABL kinase domain mutations. Our results suggest that the observed high rate of resistance in our patients is not due to ABL kinase mutations. However, it must be kept in mind that direct sequencing has a limited sensitivity and if the mutated ABL is less than 30% of the total ABL domain then a low level mutation could have been missed. An alternative approach such as High Resolution Melting (HRM) technology accompanied with sequencing, single nucleotide polymorphisms (SNPs) assay to detect and quantify low level mutations is needed to confirm their absence and to rule in/out the existence of mutations or SNPs that might be uniquely contributing to our patients resistance to Imatinib. If absence of mutations proves to be true, then other contributing mechanism(s) should be sought, such as BCR-ABL over expression, increased levels of P-glycoprotein (Pgp), up regulation of plasma protein alpha1 acid glycoprotein (AGP) and/or the existence of intrinsic factors that reduce the bioavailability of Imatinib.

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PROGNOSTIC VALUE OF CYTOGENETIC ABNORMALITIES ASSOCIATED TO PHILADELPHIA CHROMOSOME IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA TREATED WITH TYROSINE KINASE INHIBITORS

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Background. Prognosis of chronic myeloid leukemia (CML) has changed dramatically in the last decade since the introduction of tyrosine kinase inhibitors (TKI). However, there are still an important number of patients with unsatisfactory response to this therapy. Cytogenetic abnormalities associated to Ph chromosome have been classically considered as a worse prognosis indicator. Their importance in CML patients with TKI therapy must be further investigated, e.g. the expert board of the European LeukemiaNet (ELN) has dropped off the presence of $9q^+$ at the time of diagnosis as an adverse prognosis marker, but on the other hand has considered the appearance of clonal chromosome abnormalities in Ph^+(CCA/Ph^+) in the follow-up a therapy failure. *Aims.* To analyze the prognostic value of cytogenetic abnormalities associated to Ph chromosome in patients with CML treated with TKI. *Methods.* We analyzed the response to Imatinib 400 mg in 76 patients attended in our center (50% in first line, 50% in second or more lines). Sokal index at diagnosis was low in 47%, intermediate in 43% and high in 10% of patients. All patients underwent bone marrow karyotyping at diagnosis and at the 3th, 6th, 12th, and 18th month, and yearly after this point in patients that maintained complete cytogenetic response. Clonal evolution (CE) is definite as the development of non-random chromosomal abnormalities (CCA) in addition to the Ph chromosome. BCR-ABL transcripts were quantified by rt-PCR every 3 months in peripheral blood. In those patients with an inadequate response, we examined the Ph chromosome mutations as well as the plasmatic levels of Imatinib. *Results.* The responses according to ELN criteria were: failure in 21%, suboptimal response in 17% and optimal response in 62% of patients. The frequency of CCA at the time of diagnosis was 9%. In the follow-up CE was developed in 18%. The frequency of therapeutic failure was similar in patients with and without CCA at the time of diagnosis (29% *vs.* 39%; RR 0.85, 95% CI=0.51 to 1.41), but the development of CE was associated to a higher percentage of therapeutic failure in the complete cohort (71% *vs.* 31%; RR 2.42, 95% CI=1.04-5.65). Development of CCA in patients who achieved a major molecular response (MMR) in the firsts 6 and 12 months after diagnosis was low (0 and 16% respectively). Nevertheless, these subgroups were too small to obtain statistical significances (RR for CCA at 12th month 0.83, 95% CI=0.69-1.00). *Conclusions.* The prognosis impact of cytogenetic abnormalities is still an ongoing issue. In our series, CCA is not a prognosis marker at the time of diagnosis but implies a worse prognosis when it develops in the follow-up time. There is a tendency against the development of CCA when the MMR is achieved in the firsts 6 and 12 months after diagnosis.

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UNFAVOURABLE OUTCOME OF CML PATIENTS CARRYING COMPLEX VARIANT CHROMOSOMAL TRANSLOCATIONS TREATED WITH TYROSINE KINASE INHIBITORS

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Background and Aims. Cytogenetic variants of the Philadelphia (Ph) chromosome can be observed in 5-8% of patients diagnosed with Chronic Myeloid Leukemia (CML), and usually involve at least one chromosome other than 9 and 22. Despite the genetically heterogeneous nature of these alterations, available data indicate that CML patients displaying complex variant translocations (CVTs) do not exhibit a less favorable outcome as compared to individuals presenting conventional Ph-positive CML. *Patients and Methods.* We report our experience with 10 CML patients carrying CVTs among 153 newly diagnosed cases followed at our Institution between January 2003 and June 2009. All patients included in this series were diagnosed as CML in chronic-phase and their main clinical characteristics are listed in Table. Clinical, cytogenetic and molecular responses to TKIs were rated according to the European LeukemiaNet (ELN) 2006 guidelines. Peripheral blood samples were used for BCR-ABL determination by quantitative real-time polymerase chain reaction according to the suggested recommendations and all BCR-ABL transcripts were measured according to the International standardized Scale (IS). Cytogenetic responses (CyR) were evaluated on no less than 20 marrow cell metaphases with standard G-banding techniques. *Results.* In our series only two CML patients exhibiting CVTs achieved an optimal response to tyrosine kinase inhibitors (TKI) treatment. The remaining eight patients obtained either a suboptimal response or failed TKIs therapy. Statistical analysis revealed that patients carrying CVTs at diagnosis exhibited a poor clinical outcome as compared to CML patients without CVTs (P=0.02). *Conclusions.* These findings suggest that the presence of CVTs at diagnosis might confer an unfavorable clinical outcome as these genetic alterations might be markers of genomic instability and indicate a higher likelihood of disease progression.

Table.

N	Sex	Age	Sokal score	Euro score	CML phase	BCR-ABL transcript	Karyotype
1	M	29	LOW	LOW	CP	e13a2(b2a2)	46, XY, t (7;9;22)
2	M	44	INT	LOW	CP	e14a2(b3a2)	46, XY, t (2;6;9;22)
3	M	35	LOW	LOW	CP	e14a2(b3a2)	46, XY, t (9;22;15)
4	M	59	INT	HIGH	CP	e13a2(b2a2)	46, XY, t (9;11;21;22)
5	F	64	INT	LOW	CP	e13a2(b2a2)	46, XX, t (9;17;22)
6	M	55	LOW	INT	CP	e14a2(b3a2)	46, XY, t (9;22;8)
7	M	38	LOW	LOW	CP	e13a2(b2a2)	46, XY, t (9;22;8)
8	M	69	INT	INT	CP	e13a2(b2a2)	46, XY, t (4;22;9)
9	M	74	INT	HIGH	CP	e13a2(b2a2)	46, XY, t (9;22;12)
10	M	66	LOW	LOW	CP	e13a2(b2a2)	46, XY, t (9;22;17)

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PRE-EXISTING AND ACQUIRED MORE FREQUENT KD MUTATIONS OF BCR-ABL IN PRIMARY AND SECONDARY RESISTANT IMATINIB-TREATED CML PATIENTS

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Background. Mutations of the BCR-ABL tyrosine kinase domain are the major cause of resistance to imatinib in patients with chronic myeloid leukemia. However, the incidence of KD mutations in imatinib-naive patients is unknown. It is not known whether KD mutant clones that are detectable prior to therapy are selected in the presence of imatinib, and whether they may predict for therapy outcome. *Aims.* We sought to investigate the occurrence and dynamics of 5 common KD mutations (T315I, F311L, M351T, E255K, E255V) in imatinib-treated patients lacking predicted therapy respone (by ELN criteria). *Materials and Methods.* Out of 34 imatinib treated CML patients at our institution, 8 of them showed primary resistance to imatinib with inadequate CgR and consistently high levels of BCR-ABL transcripts monitored by real-time PCR. Two patients showed secondary resistance with loss of CCgR and loss of MMolR. All patients had intermediate or high Sokal score at diagnosis. Using ASO-PCR we investigated the prevalence and the evolution of 5 common BCR-ABL KD mutations in pretherapeutic leukemic samples and follow-up samples collected throughout imatinib therapy of those patients. *Results.* In all patients with primary resistance KD mutation was detected in imatinib-naive pretherapeutic leukemic samples. F311L was detected in 3/8 (37.5%) and M351T in 6/8 (75%) patients, with 2 of them harboring dual mutation F311L/ M351T. One patient had no detectable mutations, but acquired M351T after 3 months of imatinib. After 24 months of imatinib therapy identical mutation status was identified in 4/8 patients (50%). Three patients acquired additional mutation (F311L, E255K, E255V, one each). Patients harboring dual mutations (n=4) had approximately 10 times higher BCR-ABL transcripts compared to patients with single KD mutation. Two patients with secondary resistance showed no mutations at the time of diagnosis. At the time of loss of MMolR, they both showed M351T. With the increase of the imatinib dose, one of them achieved MMolR again. T315I mutation was not detected in any patient. *Conclusions.* We conclude that: (1) in patients with primary resistance KD mutations occur prior to imatinib therapy, and are selected during therapy, indicating that the mechanism of resistance was in operation since the beginning of therapy, (2) mutations may also be acquired during disease progression and imatinib therapy through associated genetic instability, (3) mutation (e.g. M351T in

our patient) is accompanied by molecular progression of the disease, (4) the sensitive detection of mutated clones may provide clinical benefit leading to early therapeutic modifications.

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NILOTINIB SAFETY AND RESPONSES ANALYSIS IN RUSSIAN PATIENTS WITH CHRONIC MYELOID LEUKEMIA (CML) FROM ENACT(EXPANDING NILOTINIB ACCESS IN CLINICAL TRIALS) STUDY

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Background. Nilotinib is a potent and highly selective BCR-ABL kinase inhibitor, approved for the treatment of patients (pts) with Philadelphia chromosome-positive chronic myelogeneous leukemia (Ph^+ CML) in chronic phase (CP) and accelerated phase (AP) who are resistant or intolerant to prior therapy, including imatinib. ENACT study was initiated as a global expanded access program to obtain additional safety information in CML pts in a clinical practice setting outside of a registration study. This report focuses on a subset of patients enrolled in Russian Federation. *Methods.* Adult patients with imatinib resistant or -intolerant Ph^+ CML-CP, AP or BC were admitted to the study. Pts received nilotinib 400 mg twice daily (BID). Pts who required dose reduction to 400 mg once daily due to toxicity were allowed to have a dose re-escalation to 400 mg BID after resolution of the adverse events (AEs). *Results.* A total of 130 pts were enrolled in the ENACT study between 01/2006 and 10/2008, including 102 CP pts (79%), 20 AP pts (15%) and 8 BC pts (6%). The median age of all pts was 46 years; 71% were imatinib-resistant, and 29% were imatinib-intolerant. The most common prior antineoplastic therapy (other than imatinib) was hydroxyurea, interferons, cytarabine. At study completion, 72 pts (71%) at CP, 11 pts (55%) in AP and 0 pts were continuing on nilotinib; 30 pts(29%), 9(45%) and 8(100%) discontinued treatment, 18 (18%) CP pts, 6 (30%) AP pts, and 6 (75%) BC pts discontinued due to disease progression. There is a total of 1 (1%) death during the study. Median (range) duration of nilotinib exposure was 212 (9-693) days for CP pts, 199 (24-679) days for AP pts, and 61 (19-101) days for BC pts; median average dose intensity was 785, 743 and 786 mg/day, respectively. 29 CP pts (28%), 5 AP pts (25%) and 1 BC pts (13%) had their dose reduced due to AE, while 51 CP pts (50%), 11 AP pts (55%), and 4 BC pts (59%) had their dose interrupted due to AE. Median duration of dose interruption due to AE was short (13 days for CP, days for AP and 5 days for BC pts). The most common grade 3/4 hematologic AEs suspected of being drug related were thrombocytopenia (24%, 35%, 38%) and neutropenia (20%, 17% ,38%) in CP, AP and BC pts. The most frequent non hematologic all grades AEs or lab abnormalities included rash, headache, nausea, fatigue and hyperbilirubinaemia, and all were slightly lower in Russian patients compared with the overall ENACT population. No patients discontinued treatment due to pleural effusion and there was no incidence of QTcF prolongation > 500 msec. Overall, complete hematological responses (CHR) rates were 55% in CP, 35% in AP and 0% in BC pts while major cytogenetic responses (MCyR) rates were 31% in CP, 20% in AP and 13% in BC pts. *Conclusions.* Analysis of Russian patients' sub-population demonstrated what nilotinib is well tolerated and effective in heavily pretreated CML patients.

Table. Patient disposition (n = 130).

	CML-CP n = 102 n(%)	CML-AP n = 20 n(%)	CML-BC n = 9 n (%)
Patients continuing nilotinib at the end of protocol treatment	72 (71)	11 (55)	0
Discontinued	30 (29)	9 (45)	8 (100)
Progression	18 (18)	6 (30)	6 (75)
Adverse event	10 (10)	2 (10)	1 (13)
Death	1 (1)	0	0
Others*	1 (1)	1 (5)	1(13)

* Includes patients who withdrew consent and abnormal laboratory value(s).

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DETERMINATION OF BCR-ABL ELEVATION LEVEL THAT CORRESPONDS TO MUTATION DETECTION IN CHRONIC MYELOID LEUKEMIA PATIENTS TREATED WITH TYROSINE KINASE INHIBITORS

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Background. BCR-ABL mutations are known to be one of the most frequent causes of acquired resistance to tyrosine kinase inhibitors (TKI). In some cases they lead to the treatment failure. Aim. To evaluate *BCR-ABL/ABL* threshold rising level that clearly predicts presence of *BCR-ABL* mutation. *Methods.* In current study 48 patients (pts) with suboptimal response or treatment failure according to the European LuekemiaNet criteria (M. Baccarani *et al.*, 2009) were included. Conventional cytogenetic analysis was performed every 6 months. Quantitative measurement by real-time PCR of *BCR/ABL* transcript level was done every 3 to 6 months after first year of therapy. 28 pts (58.3%) had lack of complete cytogenetic response (CCyR) by 12 months of IM therapy. Loss of response was detected in 12 pts (25.0%) achieved CCyR only and in 8 pts (16.7%) achieved both CCyR and major molecular response (MMR). mRNA was isolated from peripheral blood leukocytes and analyzed for point mutations in the *BCR-ABL* kinase domain by reverse transcription-polymerase chain reaction and direct sequencing at ABI Prism 3130 genetic analyzer. Our primers covered area spanning from a3 to a11 ABL exons of BCR-ABL gene, including the whole *BCR-ABL* kinase domain and flanking regions. Elevation of *BCR-ABL/ABL* was calculated by subtraction of *BCR-ABL/ABL* value at the time point (TP) prior to mutation screening to the *BCR-ABL/ABL* value at TP where mutation was detected. Threshold level was defined by ROC curve analysis. Positive and negative predictive values (PPV, NPV), sensitivity, specificity and overall correct prediction (OCP) were calculated. *Results.* Mutations in *BCR-ABL* gene were detected in 17 of 48 cases, including 3 different point mutations in P-loop, 3 mutations in imatinib-binding site, 2 mutations in A-loop, and 1 mutation outside the tyrosine kinase domain (TKD). Number of pts and mutations are shown in table. 14 (82.4%) out of 17 pts did not achieve CCyR. Median time from imatinib treatment start to mutation detection was 29 months (range 4-48 months). ROC curve analysis determined that increasing of *BCR-ABL/ABL* level in 5.5 times corresponds to 100% of NPV. In spite of excellent specificity (100%) other parameter of diagnostic performance such as sensitivity, PPV and OCP were relatively low (30.8%, 35.7%, 50% respectively). *Conclusions.* Mutation detection can be concerned as screening methods where NPV is the most valuable parameter. Thus *BCR-ABL/ABL* elevation in 5.5 times helps not to omit every single patient with *BCR-ABL* mutations.

Table. Number and localization of detected mutation.

Mutation localization		Number
P-loop	M244V	3
	G251A	1
	Y253H	4
Imatinib-binding site	T315I	4
	F317L	1
Catalytic domain	M351T	1
A-loop	V379I	1
	H396R	1
Outside TKD	E450G	1

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SURVIVAL, CYTOGENETIC AND MOLECULAR RESPONSES IN CHRONIC MYELOID LEUKEMIA CHRONIC PHASE PATIENTS TREATED BY IMATINIB: 9-YEAR FOLLOW-UP DATA FROM POPULATION ST-PETERSBURG AND LENINGRAD REGION DATABASE

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Background. During the last decade Imatinib (IM) become the first line treatment for Chronic Myeloid Leukemia (CML) patients. There are few data about the efficacy of IM beyond clinical trials without patient selection based on strict eligibility criteria. *Aims.* The aim of this study was to analyze survival, cytogenetic and molecular responses in CML chronic phase (CP) patients treated by IM in different centers in Saint-Petersburg and the Leningrad region in 9 years follow-up period. *Patients and methods.* In our database there are 234 CML CP (ELN criteria) pts, who have been treated with IM since 2001. Before IM the pts were pretreated with hydroxyurea (79.5%), interferon-alfa with or without hydroxyurea (48.3%) and busulfan (6.8%). There were 133 (56.8%) pts in early CML CP (disease duration before IM ≤12 months) and 101 (43.2%) in late CP (>12 months). *Results.* The median time before IM for the whole group was 6.75 months (3 days - 243 months), for early and late CP patients it was 1.7 months (3 days - 12 months) and 38 months (13.3 - 243 months) respectively. The median time of IM therapy was 32 months (0.33 - 85 months) for the whole group, 28 months (1-82 months) in early and 41 months (0.33 - 85 months) in late CP patients respectively. The estimated overall survival (excluding CML unrelated deaths) by 9 years was 85%. The rate of CML related death was 8.3% (19/229). The probability of complete cytogenetic response (CCyR) in the whole group was 82%. In early CP the probability of CCyR was definitely higher, than in the late phase: 92% *vs.* 75%, P=0,003. There was no difference between patients who had been pretreated by interferon and those who had not (83% and 83%, P>0.5). No significant differences between the probability of CCyR in patients with or without clonal evolution before imatinib therapy (86% *vs.* 68%, P>0.5) was found. The rate of CCyR loss was 29.7%. It was equal in patients treated with or without interferon before IM (29% *vs.* 30%, P>0.5). The rate of major (MMR) and complete (CMR) molecular response was 50% (59/117) and 37% (45/117) respectively. It depended on time to CHR and CCyR. The probability of MMR by 5 years was 64% *vs.* 0% with or without CHR by 3 months (P<0.05), 64% *vs.* 48% (P<0.05) with or without CCyR by 12 months. Imatinib therapy was stopped in 34.2% (80/234) of patients. The reasons for discontinuation were: resistance - 66.25% (53/80), intolerance -15% (12/80), death from causes other than CML - 6.25% (5/80), stem cells transplantation - 1.25% (1/80), 3.75% (3/80) - patient decision, 7.5% (6/80) - unknown reason/loss to follow-up. *Conclusions.* IM therapy was very effective in patients, treated in outpatients departments of Saint-Petersburg and the Leningrad region. The most patients are alive and still on IM therapy. No significant difference in achievement or loss of CCyR for patients who were and who were not pretreated by interferon was revealed. The probability of MMR was higher in patients with early CHR and CCyR.

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IMATINIB IN CLINICAL PRACTICE IN TREATMENT OF PATIENTS WITH CML. RESULTS OF LARGE COHORT FROM SINGLE UNIVERSITY CENTER IN SOUTHERN EUROPE

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Prospective trials showed high efficacy of imatinib in treatment of CML, but few data are available about non trial clinical patients. We analyze efficacy of IM in treatment of large cohort of CML patients from 2002 to 2009, from database of Clinical Center of Serbia. *Patients.* In the period of >7 years, 90 patients with CML was treated by regular IM (health insurance), and 32 patients were enrolled in CELSG "ISTAHIT" trial between 2004-2006. *Results.* Mean age was 47.4 yrs (17-76). Mean follow up was 35 mths (9-170). Four patients were treated in BC-CML, with poor outcome. 86 patients were treated either as 1st line IM (63 pts, 70%) or 2nd line chronic phase, CP (14), or accelerated phase AP (9 pts). We found high proportion of high Sokal (39%) and Hasford (12%) scores. We had 3 Ph-, bcr-abl+ patients (3%). All patients started with 400mg IM with subsequent escalation in non optimal response according to National guidelines based on ELN 2006&NCCN 2007. Only 3/9 patients in AP achieved major CgR at 6m, but 2/9 achieved CCgR at 12 and 24m. From patients treated in CP, 12/13 patients achieved at least MCgR at 6m with 2nd line IM, and 53/61 evaluated (86%) achieved at least MCgR at 6m, with 70% of CCgR with 1st line IM. At 12 months, all 12/13 pts treated with 2nd line IM achieved CCgR, and 41/47 patients evaluated (87%) were in CCgR on 1st line IM. 2 of 3 Ph negative pts achieved MMR. There was no significant difference between 1st and 2nd line IM treatment. Seven patients (9%) in CP had treatment failure or progression (events), and 5 (6%) experienced toxicity resulting in IM withdrawal. Concerning survival, 2 pts in AP with CCgR continues IM like CP patients, 2 patients were transplanted, and 5 died due to progression. From patients in CP, 74 (96%) is alive, with OS5y 97%, with no difference between 1st and 2nd line IM. Concerning progression free survival, PFS, we have found difference between 1st and 2nd line IM treatment but it would be worth to note that 2nd line IM treatment group was small, and also had at least 1.5 year of stabile chronic phase before IM treatment. We have not found significant difference in survival of CP patients concerning Sokal risk but in general patients with high risk did slightly worse (OS5y 93%) than low and particularly intermediate risk (OS5y 100%). There was a slight difference in PFS with PFS5y for Sokal low risk 95%, and 70% for intermediate and high risk group. We have not found that difference concerning Hasford score. *Conclusion.* Our results are in concordance with previous published data from clinical trials and also some cohort data. Introduction of National guidelines based on ELN proposals, positively affect CML treatment and IM has proven to be efficient treatment for all patients in CP, but for patients in AP it would be necessary to find different strategies.

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HIGHTROUGHPUT BLOOD LEVEL TESTING OF IMATINIB BY FLOW INJECTION ANALYSIS - TANDEM MASS SPECTROMETRY

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Background. Imatinib (Glivec) is a selective tyrosine kinase inhibitor currently used for the treatment of Philadelphia chromosome-positive chronic myeloid leukemia (CML) and for other malignant pathologies. Usually it is determinated by liquid chromatography coupled with (tandem) mass spectrometry, but the analyses are time consuming. *Aims.* The aim of this work was to develope of new rapid and sensitive flow injection analysis with tandem mass spectrometry detection for determination of imatinib (IM) in plasma. *Methods.* Plasma was deproteinated by methanol with added internal standard (D8-deuterated imatinib), shaken, freezed and centrifugated. Imatinib plasma levels were measured using UHPLC UltiMate 3000 RS (Dionex) coupled with the API 4000 tandem mass spectrometer (Applied Biosystems). Mobile phase consisted of methanol and 0.1% formic acid. A triple quadrupole detector with electrospray ionization was operated in multiple-reaction monitoring mode (m/z transitions for IM 494=>394, D8-IM 502=>394; dwell time 200 ms). Our method was compared with currently accepted method (Titier K, Blood, 2005), where chromatographic separation on a reverse phase analytical column Acquity UPLC® BEH C18 1.7 µm (2.1 ×50 mm; Waters) we used. *Results.* Total analysis time of our method (45 s) was 10 times faster compared to Titier's original method. The method was fully validated and tested on plasma samples from 120 patients treated with IM. All evaluated parameters (recovery, imprecision, accuracy), Bland-Altman plot (mean difference -1.5 and SD 29.0 ng/mL) and regression analysis (y = 0.995x + 3.295; r2 = 0.997) revealed agreement of tested methods. *Conclusions.* This method is simple and high throughput, adapted to routine application and it is suitable for therapeutic monitoring of imatinib. It provides comparable results with commonly used liquid chromatography-mass spectrometry method but it is much faster and cheaper. *Acknowledgement.* The work was supported by grants NS9627-3 (Ministry of Health, the Czech Republic), MSM 6198959205 (Ministry of Education, Youth and Sports, the Czech Republic).

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RELATIONSHIP BETWEEN AN EFFECTIVE DOSE OF IMATINIB AND TROUGH LEVEL IN KOREAN CHRONIC-PHASE CML WITH REDUCED DOSE

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Background. Some patients in chronic-phase chronic myeloid leukemia (CML) receive reduced doses of imatinib due to some adverse effects in practical setting. Their clinical response was comparable with the data of other reports including the IRIS study despite low dose of imatinib. Recently, the effective trough levels of imatinib were demonstrated in some studies. *Aims.* In this study, we tried to verify the relationship between effective dose of imatinib and the trough level in Korean chronic-phase CML with reduced dose. *Methods.* Forty-eight patients having record of BSA from 2 University Hospital in Korea (Korea University Medical Center and Chonnam National University Hwasun Hospital) who treated with reduced dose of imatinib were retrospectively analyzed. Informed consent was obtained from 23 of 48 patients to measure the trough level of imatinib. This study was approved by the Ethic Committees of the participating hospitals. *Result.* Thirty-three patients (68.8%) were reduced dose to 300 mg/day within six months after starting imatinib therapy with 400 mg/day. The main reasons for dose reduction were severe neutropenia or thrombocytopenia (grade 3 or 4) (62.9%). The cumulative complete cytogenetic response (CCR) at 12 months was 62.5%. After median follow-up period of 38.6 months (7.2~88.6), 36 patients (75.0%) have sustained CCR. The mean dose of imatinib/day of all patients were 328.6±34.2 mg (208.0 ~ 394.2). The mean BSA was 1.58±0.16 (1.28~1.97) with significant difference by gender (P<0.001)(male; 1.70±0.13 *vs.* female; 1.49±0.12). Also, the mean dose of imatinib/BSA of male patients was significantly lower than female patients with low BSA (P = 0.034) (male; 199.6±18.5 mg/m^2 *vs.* female; 216.3±30.1 mg/m^2). The mean dose of imatinib/BSA was significantly higher in patients achieving cumulative CCR at 12 months compared with those couldn't achieving efficacy (P<0.032)(215.2±23.6 mg/m^2 *vs.* 196.5±31.4 mg/m^2), although the gender, the mean dose of imatinib and BSA didn't show the difference. We measured the trough level of imatinib among 23 patients. The cumulative complete cytogenetic response (CCR) at 12 months was 73.9%. The mean trough levels of imatinib were 1370±708 ng/×(585~3940) with the mean dose of imatinib/day, 333.5±23.4 mg. The trough levels of imatinib have a significant correlation with the dose of imatinib/BSA (r2 = 0.268). The mean trough level of imatinib in patients with cumulative CCR at 12 months was relatively higher without statistically significance (P=0.097). After median follow-up period of 35.2 months (7.2~88.6), 20 patients (87.7%) have sustained CCR. *Conclusion.* The mean dose of imatinib/BSA was associated with the efficacy of reduced dose of imatinib in Korean chronic-phase CML. Although we didn't show that significant relationship between the trough levels of imatinib and effective outcomes, the trough levels of imatinib have a significant correlation with the mean dose of imatinib/BSA. Considering the smaller BSA of Korean patients, this result suggest that the reduced dose of imatinib could maintain effective trough level and excellent clinical outcomes for the threatment of chronic-phase CML in Korea.

1323

HEMATOLOGIC AND MOLECULAR RESPONSE EVALUATION OF PATIENTS WITH CHRONIC MYELOID LEUKEMIA TREATING WITH INDIAN GENERIC TYPE OF IMATINIB

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Background. Imatinib mesylate is used in chronic phase of chronic myeloid leukemia patients to induce apoptosis and inhibit cell proliferation. *Aims.* Hematologic and molecular responses of these patients to Indian generic type (Imatib) were studied, since 95% of Iranian CML patients use this drug as a first-line therapy due to it's cheaper price (about 12 times cheaper). *Methods.* Thirty patients with chronic myeloid leukemia who were treated with Imatib were analyzed in this investigation (informed consent was obtained). Physical examination, CBC test and peripheral blood smear observation was used for hematologic response determination. Molecular response was also examined through quantitative assessment of BCR-ABL fusion gene expression by Real Time RT-PCR. Also, the correlation of molecular and hematologic responses with patient's age and sex, dose and duration of Imatib consumption was analysed statistically. *Results.* 90% of patients showed hematologic response which did not have any significant correlation with patient's age and sex , dose and duration of Imatib consumption (P>0.05). While, 46.7% of patients showed complete molecular response, 43.3% of patients showed partial molecular response and 10% of patients showed no molecular response to Imatib. A significant reverse correlation between molecular response type and age (P<0.05) was observed. In contrast, there was no significant correlation between molecular response type and patient's sex, dose and duration of Imatib consumption (P>0.05). *Conclusions.* In this cross sectional study, results of molecular and hematologic responses to Imatib which is comparable to more expensive Gleevec, was good and acceptable. Also, More accuracy and integrity of patient's molecular response due to higher sensitivity of Real Time RT-PCR technique, shows the necessity of molecular response determination in addition to hematologic response for better assessment of patient therapeutic situation.

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CHRONIC MYELOID LEUKEMIA IN THE IMATINIB ERA: THE PREDICTIVE VALUE OF OPTIMAL RESPONSE AT 12 MONTHS OF THERAPY ON ACHIEVING MOLECULAR RESPONSE AND DISEASE PROGRESSION

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Background. Imatinib as first line treatment for chronic myeloid leukemia (CML) has improved significantly the overall survival, progression free survival and event free survival of these patients. Achieving complete cytogenetic response (CCyR) is nowadays one the primary objectives of the therapy. *Aims.* Compare the evolution of patients with CML who achieved CCyR at 12 months of therapy (optimal responders) with those with those with less than this response (non-optimal responders).

	Optimal responders (n=46)	Non-optimal responders (n=28)
CHR Median time to CHR	46 (100%) 1 mths (1-4)	28 (100%) 1.5 mths (1-4)
CCyR Median time to CCyR	44 (95.7%) 6 mths (3-12)	11 (39.3%) 16 mths (13-24)
MMR Median time to MMR	33 (71.7%) 16 mths (5-48)	10 (35.7%) 38.5 mths (24-61)
CMR Median time to CMR	16 (34.8%) 25.5 mths (12-63)	2 (7.1%) 39 mths (30-48)
Disease progression	0	2 (7.1%)
Therapeutic discontinuation (switch to 2nd generation TKI)	1 (intolerance)	9 (intolerance/resistance)
Mortality	0	3 (10.7%)

CHR: complete hematologic response; CCyR: complete cytogenetic response; MMR: major molecular response; CMR: complete molecular response; TKI: tyrosine kinase inhibitor

Methods. From May 2001 to March 2010, 88 patients with the diagnosis of CML were treated with Imatinib at our institution. Of these, 74 had a follow-up period longer than 12 months (median = 49 months), with a median age of 58.5 years (20-88). 10 patients were at late chronic phase, with a median time from diagnosis to start of Imatinib of 42,5 months (15-197). Imatinib dosage: 400mg (chronic phase), 600-800mg (accelerated/blastic phase). *Population characterization.* - optimal responders (n=46) - median age at diagnosis 53.5 years (14-78), 23 male/23 female, 42 (91.3%) in chronic phase (Sokal score: low risk 50.0%, intermediate risk 35.7%, high risk 14.3%), 2 (4.3%) in accelerated phase, 2 (4.3%) in blastic phase. 2 patients were in late chronic phase, with a median time from diagnosis to start of Imatinib of 50 months (22-78). Median follow-up 42.5 months (12-105). - non-optimal responders (n=28) - median age at diagnosis 53.5 years (19-81), 14 male/14 female, 26 (92.9%) in chronic phase (Sokal score: low risk 23.1%, intermediate risk 46.1%, high risk 30.8%). 8 patients were in late chronic phase, with a median time from diagnosis to start of Imatinib of 42,5 months (15-197). Median follow-up 60 months (14-105).

The hematologic, cytogenetic and molecular responses were evaluated according to the European LeukeamiaNet recommendations, as well as the development of intolerance/resistance to Imatinib, disease progression and mortality. *Results.* Figure 1. *Conclusions.* The achievement of cytogenetic and molecular responses is associated with an improved event free survival and progression free survival. Patients who fail to have CCyR at 12 months of therapy with Imatinib are less likely to achieve MMR, and have a higher risk of losing their response and of progression to advanced stages of the disease. These results verify the predictive value of obtaining an optimal response at 12 months.

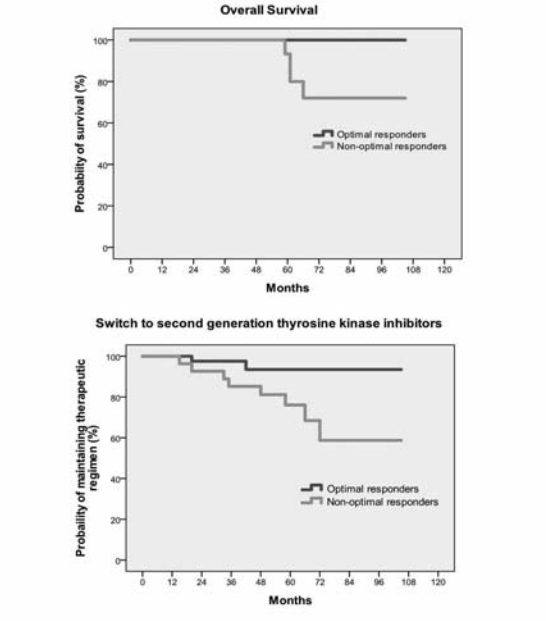

Figure 1.

1325

CUPLIKE NUCLEI IN ACUTE MYELOID LEUKEMIA: A SPECIFIC ENTITY WITH INCREASED FLT3 ITD AND NPM1 MUTATION

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Background. Cuplike Nuclei blasts have been reported in a small portion of patients (pts) presenting with acute myeloid leukemia (AML). Recent reports showed the close relationship with molecular abnormalities including the gain of FLT3 ITD and NPM1 mutation in a higher percentage than other subtypes. There is little data on the clinical features and outcome of patients with this entity. *Aims.* Describe the clinical outcome of patients with cuplike nuclei blasts. *Methods.* We searched the UT MDACC database for patients with myeloid malignancies with cuplike nuclei morphology (ie, ≥10% blasts with nuclear invagination in ≥25% of nuclear size). Pts were divided into two groups: *de novo* AML (group 1) and relapsed AML or MDS (group 2). Three- or 4-color flow cytometry immunophenotypic analysis was performed on BM aspirates. FLT3 and NPM1 mutations were investigated by polymerase chain reaction followed by sequencing on an ABI Prism 3100 or 3130 Genetic Analyzer. Kaplan-Meier method was used to estimate survival using Statistica 6 software. All patients signed an approved consent. *Results.* Forty-eight pts and 16 pts were identified at UTMDACC between 1998-2009 in groups 1 and 2, respectively. Group 1 had a median age 60 years (range 17-81), 56% were female, and more frequent of M1 morphology (44 pts, 92%). Group 2 had a median age of 62 years (25-76), slight female predominance (56%) and all AML pts classified as M1; 2 pts had a diagnosis of MDS (RAEB). Diploid cytogenetics was more frequent in group 1 *vs.* group 2 (65% *vs.* 44%). Both groups had elevated WBC (median, 44.1 and 35×10^9/L×10^9/L), D-Dimer (2001 and 892) and peripheral blasts percentage (86% and 88%) at start of therapy. In group 1, 27 (56%) pts lacked CD34 expression and 16 (33%) lacked HLA-DR. Ninety four percent of pts with no HLA-DR expression lacked CD34 too. Among evaluable pts, 21/36 (58%) in group 1 and 7/9 (78%) in group 2 had FLT3 ITD. Four out of 36 pts in group 1 and none in group 2 had FLT3 TKD mutation. NPM1 mutation was found in 9/11 (82%) in group 1, 8 (89%) of them with concomitant FLT3 ITD/TKD. CR was achieved in 55% and 60% in groups 1 and 2, respectively. IA based chemotherapy resulted in CR in 16/21 (76%) in group 1 and 8/9 (89%) in group 2. Median overall survival was 275 days for pts in group 1 and not reached in group 2. Of 12 pts who died within 35 days, 7 had molecular testing, and all had FLT3-ITD. *Conclusions.* Cuplike nuclei AML is characterized by frequent association with mutated NPM1 and FLT3 ITD/TKD, absence of both CD34 and HLA-DR, and a high frequency of FAB M1 morphology. This entity is characterized by a poor outcome.

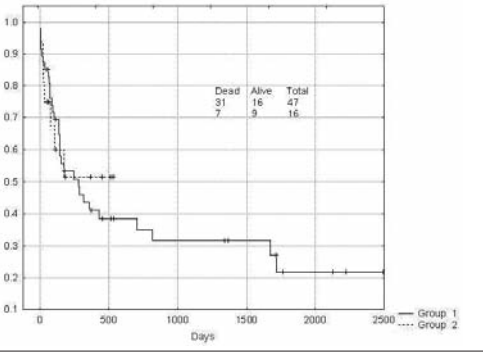

Figure. Overall Survival for group 1, 2.

1326

MANAGEMENT AND OUTCOME OF CML PATIENTS: A TUNISIAN MULTI-CENTER STUDY ABOUT 116 CASES

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Aims. Imatinib was the first treatment for chronic myeloid leukemia (CML) that specifically targeted the causative BCR-ABL transcript, producing high response rates in most patients. In this multicentre study, we retrospectively evaluate cytogenetic and molecular response to Imatinib and analyze the predictive resistance factors and their impact on event free survival (EFS) and overall survival (OS). *Methods.* From July 2002 to June 2009, 116 CML patients were included: 104 in chronic phase (CP) and 12 in accelerated phase (AP). Median age was 44 years (13 - 76 years). Sokal score, calculated in 110 cases, was high in 21 patients (18%), Intermediate in 53 patients (46%) and low in 36 patients (31%). First-line treatment in all patients was Imatinib 400mg/day, initiated at median time of 3 months (1-12months). ELN criteria were used for response evaluation. *Results.* Complete cytogenetic response (CCyR) was achieved in 76% of cases (n=88) with a median of 12 months (3-36 months). Molecular monitoring was performed in 80% of patients (n=93). Major molecular response (MMR) was achieved in 62% of them (n=58) with a median of 18 months (6-48 months). 33% of patients (n=38) were in optimal response (OpR), 46 % (n=53) in suboptimal response (SubOpR) and 21% (n=25) in primary resistance (PR). Imatinib dose escalation benefit to 72% of patients in PR (18/25), only five of them did achieve a CCyR. 11% (n=13) of patients had a secondary resistance and 85% (11/13) of them benefit of dose escalation. No other patient eligible for escalation (SubOpR: n=53) did benefit of IM increase. Predictive factors of CCyR were Sokal score and treatment discontinuation (toxicity or non adherence). Sokal score was the only predictive factor of MMR. The 5 years EFS was 79%. EFS varied according to the CCyR at 6 months (92% *vs.* 58%, P=.00001), the initial disease status (CP: 87% *vs.* AP: 58%; P= .002) and the initial response (PR: 87% *vs.* OpR-SubOpR 68%; P=.008).The 5 years OS was 88%. Only CCyR had a significant impact on OS. MMR had no significant impact on EFS or OS. *Conclusions.* In our study we note that management of resistances and suboptimal responses was not harmonized between the different centers. Despite this difficulty and even in lack of second generation TKI: EFS, OS and rate of CCyR are hopeful and may be compared to those reported in the different publications.

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ANALYSIS OF BCR-ABL KINASE DOMAIN MUTATIONS IN HUNGARIAN PATIENTS WITH IMATINIB RESISTANT CHRONIC MYELOID LEUKEMIA AND PHILADELPHIA POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA

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Background. Standard therapy of Philadelphia (Ph) positive chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL) is the targeted BCR-ABL tyrosine kinase inhibitor (TKI) imatinib mesylate, but mutations in the BCR-ABL tyrosine kinase domain (TKD) may cause imatinib resistance. The second-generation TKIs, dasatinib and nilotinib offer high likelihood of therapeutical success for imatinib resistant patients. *Methods.* We performed mutation analysis of 69 imatinib resistant CML and 2 ALL patients with direct sequencing of BCR-ABL TKD after a two-step nested PCR. In addition to imatinib, 49 of 71 patients also received second generation TKI, nilotinib or dasatinib. Mutation analysis was performed in nilotinib and dasatinib resistance as well. *Results.* In case of 21/71 (30%) imatinib resistant patients 12 BCR-ABL TKD mutations (aa. change M244V, G250E, Y253H, E255V, D276G, E279K, T315I, M351T, F359I/V, L384M, L387M) were detected, with two patients having double mutation. Mutations were found in 32% of chronic-phase (CP) patients, 36% of accelerated-phase (AP) patients and 80% of blast crisis (BC) patients. In CP patients treated with imatinib, frontline mutations were less frequent compared to CP patients treated with imatinib post-IFN failure (10% *vs.* 42%, respectively). T315I mutation was detected only in ALL, but not in CML CP in imatinib resistant patients.The reduction of BCR-ABL/ABL ratio during nilotinib treatment (n=29) did not differ between imatinib-resistant patients with or without mutation on imatinib (baseline mutation), except for cases with mutations E255V, Y253H and F359V/I. In our series, F359I mutation proved to be imatinib resistant similarly to F359V/C. There were not enough cases with baseline mutation among dasatinib treated patients (n=20) to perform this comparison. In case of second generation TKI resistance, the spectra of mutations has changed and less types of mutations were detected. In cases of 7/15 nilotinib-resistant patients, the following new mutations occured: Y253H, T315I, F359V and the following mutations persisted: Y253H, M351T, F359I; one patient had double mutation: Y253H+F359V. 2/14 dasatinib-resistant patients developed new mutations (T315I and T315+E279K). During second generation TKI treatment, the T315I mutation emerged not only in BC but in CP and AP. *Conclusions.* The frequency and type of TKD mutation depends on disease phase and TKI applied. Screening for BCR-ABL TKD mutations is recommended in TKI resistance before changing TKI, because the presence of different mutations may influence the selection of TKI and the therapeutic response.

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EFFICACY OF NILOTINIB VERSUS IMATINIB IN ADULT PATIENTS WITH PH+ CHRONIC MYELOID LEUKEMIA IN EARLY CP WHO HAVE A SUBOPTIMAL MOLECULAR RESPONSE TO IMATINIB (RE-NICE MULTICENTER STUDY)

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Background. Achievement of major molecular response (MMR) is important in imatinib therapy as it appears to predict for long-term progression-free survival. In IRIS study, progression-free survival was higher for patients who achieved both complete cytogenetic response (CCyR) and MMR, compared to patients who had CCyR but no MMR and patients who did not achieve CCyR. Compared to standard dose of imatinib, higher doses of imatinib are expected to yield higher CCyR and MMR rates, and nilotinib also produces high CCyR and MMR rates in patients with chronic phase (CP) CML who are resistant to imatinib. *Aims.* In this study, the efficacy of nilotinib at 400 mg BID was compared with imatinib at 400 mg BID in suboptimal molecular response patients who received first line imatinib therapy at a daily dose of 400 mg. *Methods.* Adult patients with Ph^+ CML early CP who have achieved a CCyR but no MMR after at least 18 months and up to 24 months (≥18 to ≤24 months) on first line imatinib therapy at a daily dose of 400 mg were enrolled. In nilotinib arm, patients received oral dose of 400 mg BID (800 mg/day), and patients received 800 mg/day administrated as 400 mg BID in imatinib dose-escalation arm. Informed consent was obtained prior to participation in this clinical trial. Primary endpoint is to evaluate the cumulative rate of MMR at 12 months, and secondary endpoints are to evaluate the cumulative rate of CMR and time to and duration of MMR and CMR. Progression-free survival and safety profiles will also be assessed as secondary endpoints. To assess the drug efficacy, cytogenetics and RQ-PCR analysis were performed at regular intervals. Baseline mutational analysis was also performed for every patient, and subsequent mutational analysis was performed in patients demonstrating either lack of response, or disease progression. *Results.* A total of 8 patients were randomized into nilotinib arm (n=4) or imatinib arm (n=4). With a median follow-up of 6 months (range, 1-9 months), all patients have maintained CCyR, and progressive decrease in BCR-ABL transcript level was observed in 7 patients except 1 patient in imatinib arm. (Figure 1). In 1 patient who showed unchanged BCR-ABL transcript level, BCR-ABL (IS%) was 0.24 at baseline and 0.25 at C9D28. Patients treated with nilotinib showed a greater depth of molecular response compared to the patients in imatinib dose-escalation arm, with 1 patient achieving MMR after 1 month of nilotinib therapy. Although toxicity was observed more frequently in imatinib dose-escalation arm, no patient required dose reduction or discontinuation of therapy due to toxicities in both randomized groups. *Conclusions.* These data show that both nilotinib and imatinib are generally well tolerated at a daily dose of 800 mg. This promising preliminary results demonstrating progressive decrease in BCR-ABL transcript level, support the potential of further clinical investigation in which the efficacy of early intervention of suboptimal molecular response using nilotinib or dose escalation of imatinib will be assessed on a large patient population and longer period of observation.

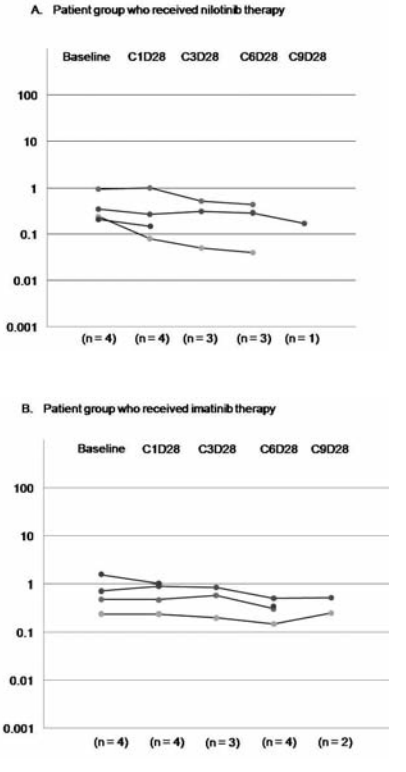

Figure 1. RQ-PCR follow-up.

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USING OF DASATINIB FOR TREATMENT IMATINIB-RESISTANT AND IMATINIB-INTOLERANT PATIENTS WITH CHRONIC MYELOID LEUKEMIA IN CHRONIC PHASE. RUSSIAN EXPERIENCE OF DASATINIB TREATMENT

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Objectives. Resistance to imatinib therapy is an unmet medical need in CML treatment. The one of pathway for decision this problem is in using of second line of TKI. The aims of this article are to describe the subanalysis and representation of subanalysis data covering dasatinib efficacy and tolerability from Russian patients who did participate in the international randomized trials Dasatinib 140 mg *vs.* imatinib 800 mg (START-R) and the dose optimization study 034. Totally there were 101 patients with dasatinib treatment in the Start-R international randomize trial and 670 patients in the 034 international dose optimization study. *Materials.* In the Russian Hematology Research Center we treated 36 (7 patients from START-R, 11 patients from 034 study and 11 patients from compassion use) adult patients with chronic phase CML with dasatinib. Median of age was 52 years (range 17-72). Median time from diagnosis to dasatinib treatment was 63 months (range 16-241). Patient was treated with hydrea, a-interferon and imatinib. Median of imatinib treatment was 0,5 months (range 0,3-33). *Results.* Duration of dasatinib treatment until March 2009 was 28 months (range 2-44). There were no cases of death during dasatinib treatment. Two patients died after discontinuing dasatinib, due to progression of CML. Complete hematological response were observed in 89% patients receiving dasatinib, major cytogenetic response in 57% patients, complete cytogenetic response in 43%, major molecular response in 25%, complete molecular response in 16%. Three patients were resistant and one patient had intolerance. Hematological toxicity 3-4 grade was described in 12(33%) patients. Non-hematological toxicity 3-4 grade was in 7 (19%) patients. Pleural effusion was diagnosed in 6(17%) patients. Dose interruptions of dasatinib were required in 15(42%) and dose reductions were noted in 15(42%) patients. The patients who had a dose reduction or a dose interruption have restarted their treatment in initial dose. Dasatinib treatment was discontinued for one patient with pleural effusion and complete molecular response is remaining during one year without any treatment. 26 (66%) patients continue therapy with dasatinib. *Conclusion.* Results from trials in Russian Hematology Research Center are the same as in international study. Dasatinib is effective and well tolerated therapeutic option for imatinib-resistant patients with chronic phase of CML.

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RENAL FAILURE ASSOCIATED TO TYROSINE KINASE INHIBITORS

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Some studies suggest that tyrosine kinase inhibitors may cause renal failure, as rare side effect of this treatment. We report here cases of seven patients, four male and three female, who developed subacute or acute renal failure in our Department during tyrosine kinase inhibitors treatment. The significant biochemical data of the patients are summarized on Table 1. Of seven patients, two patients showed mild renal failure at diagnosis, one patient developed severe renal failure after seven years of Imatinib therapy, four patient showed mild renal failure during TKIs therapy. In detail, the two patients (PA and PC) who showed mild renal failure at the time of diagnosis of CML were 71yo and 68yo respectively. Both patients received Imatinib therapy for 10 years and 5 years, respectively. They showed a gradual worsening of their renal failure during the time, until now, with needed of eritropoietin support and aproteic diet. In one of two, the renal failure didn't solve after switch to Dasatinib (April 2007) when there was the worsening of renal failure, neither with Nilotinib. They are now in CHR, CCyr and MMR. The only one patient who died (AC), received diagnosis in 1999 and he started Imatinib in 2002; he showed severe renal failure in 2009 after seven years of Imatinib therapy. He died after two months for acute renal failure. The remaining 4 patients had a median age at diagnosis of 69.7 yo (66-77). All of them received imatinib therapy for a median period of 4.5 years (3-7) at the first signs of renal failure; one of them showed mild renal failure after 6 month to switching to Dasatinib for secondary resistance to Imatinib. In this case, the renal failure didn't solve neither after the switch to Nilotinib (May 2009) for appearance of F317L mutation. All 4 patients are at moment in CHR, CCyR and in suboptimal molecular response, with mild signs of renal failure, continuing three Imatinib and one Nilotinib therapy with good compliance. Renal failure can be considered as an extremely rare adverse event of TKIs. In our cases the renal failure developed gradually, and the patients with pre-existing renal disease showed a slight worsening. It is not really clear what is the real role of TKIs in renal failure onset. Some reports suggest the role of the TKIs in inhibiting signalling pathways which may play a role in renal injury. The responsible mechanism could be the inhibition of other tyrosine kinase receptors expressed in the kidney, with a consequent tubular disfunction. The alterated metabolism of Calcium and Phosphorous might be another possible cause, as suggested by some reports. All these mechanisms could have a role when predisposing factors (age, diabetes, hypertension) or pre-existing renal failure are present. In conclusion, TKIs-related nephrotoxicity may be a relatively rare side effect in CML patients. An accurate biochemical follow-up, especially in older patients and in patients with predisposing factors, it would be useful before the treatment and during the follow up of the disease.

Table 1.

PTS	RF at diag	Time from start TKIs	UREA	Crea	Uric acid	Ca	P	K
AC	No	7 ys	60	3,2	6,4	8,7	2,8	4,0
BM	No	7 ys	91	1,4	8,6	9,6	3,5	4,5
DC	No	5ys	60	1,4	4,2	8,8	3,4	5,5
CT	No	6 mo	73	1,6	7,5	9,8	3,1	4,4
PA	Yes	NA	82	2,0	9,4	9,2	5,0	6,3
PC	Yes	NA	94	2,4	4,4	9,3	4,4	6,0
SC	No	3 ys	65	1,5	6,6	9,5	2,9	4,8

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PREVALENCE OF BCR-ABL IMATINIB RESISTANT MUTATIONS IN PORTUGUESE CHRONIC MYELOID LEUKAEMIA (CML) PATIENTS

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Background. The Tyrosine Kinase inhibitor Imatinib Mesylate (IM) is the current standard treatment in patients with in Chronic Myeloid Leukemia (CML). However, there are patients who do not respond to Imatinib or, after an initial response, develop resistance. There are several resistance mechanisms that can either be BCR-ABL dependent (genomic amplification and point mutations in BCR-ABL fusion gene) which are the main mechanism of acquired resistance or BCR-ABL independent. In these patients Dasatinb has proved to be an efficient therapeutic alternative. *Aims.* To identify the mutation frequency and mutation pattern in ninety (90) CML patients filling criteria for acquired resistance to imatinib therapy. *Methods.* BCR-ABL quantification was done according to EAC protocol. Mutation screening was done by automatic sequencing of a RT-PCR-amplified segment including the BCR-ABL Tyrosine Kinase domain. Karyotype was performed according to standard procedures. *Results.* BCR/ABL mutations were identified in thirty five patients (38.9%). In this cohort a total of 20 different IM resistant mutations (18 known and 2 new), 1 polymorphism and 1 unknown variant were identified. Seven patients had more than one BCR/ABL mutation. In the remaining fifty five (61.1%) some had complex karyotypes with complex Philadelphia variants or additional cytogenetic abnormalities (30%) and the remaining (31.1%) showed no alterations. *Summary/Conclusions.* The distribution of the mutations was: D276G in 5 cases, G250E, E255K, T315I, M351T and H396R in 4 cases, Y253H, E255V, F317L and L387M in 2 cases and mutations M244V, F359I, M388L, A397V, F416S, S438C, E450K, E453K, E459K and F486S in 1 case each. Although these results are according with the most frequently mutated amino acids reported, there are some differences such as for the D276G mutation, that in this cohort has the highest frequency and for F359 variants, which in this cohort are less represented. The polymorphism K247R was identified once, as well as the variation G383G. This last variant hasn't been described yet and was found together with the mutations H396R and A397V. This data indicate a possible variability among different populations and emphasize the fact that patients who fail IM therapy should undergo mutational analysis to define a more efficient treatment.

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THE IMPACT OF AGE ON THE EFICACY AND TOXICITY OF IMATINIB TREATMENT IN CHRONIC MYELOID LEUKEMIA

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Background. Imatinib has become the gold standard in general practice for chronic myeloid leukemia (CML). Old age has been classically considered a feature of bad prognosis for CML. Imatinib has changed dramatically the course of the disease and is forcing the clinicians to reassess the importance of previously considered indicators of prognosis. *Aims.* Evaluate the efficacy and tolerability profile of Imatinib in elderly patients (≥ 65 years) with CML, in comparison to younger patients (< 65 years). *Methods.* This is a retrospective study, including all patients treated with Imatinib in our institution, from May 2001 to March 2010 (n=88 patients). *Population characterization.* - elderly patients (n=22) - median age at diagnosis 71.5 years (65-86), 11 male/11 female, 21 (95.5%) in chronic phase (Sokal score: low risk 52.3%, intermediate risk 28.6%, high risk 19.1%), 1 (4.5%) in accelerated phase. Median follow-up 49.5 months (6-87). - younger patients (n=66) - median age at diagnosis 47 years (14-64), 32 male/34 female, 60 (90.9%) in chronic phase (Sokal score: low risk 33.3%, intermediate risk 41.7%, high risk 25.0%), 4 (6.1%) in accelerated phase, 2 (3.0%) in blastic phas. Median follow-up 38.5 months (3-105). *Therapeutic regimen.* Imatinib 400mg (chronic phase), 600-800 mg (accelerated/blastic phase). The hematologic, cytogenetic and molecular responses were evaluated according to the European LeukeamiaNet recommendations, as well as the therapy associated toxicity, the disease progression and the mortality.

	Younger patients (n=66)	Elderly patients (n=22)
CHR	66 (100%)	22 (100%)
Median time to CHR	1 mths (1-4)	2 mths (1-4)
CCyR	45 (68.2%)	15 (68.1%)
Median time to CCyR	6 mths (3-23)	8 mths (3-24)
MMR	32 (48.5%)	10 (45.5%)
Median time to MMR	18 mths (4-57)	18 mths (6-50)
CMR	13 (19.7%)	5 (22.7%)
Median time to CMR	27 mths (12-63)	30 mths (12-36)
Disease progression	2 (3.0%)	0
Hematologic toxicity (grade≥3)	4 (6.1%)	2 (9.1%)
Non hematologic toxicity (grade≥3)	3 (4.5%) - hepatic	1 (4.5%) -hepatic
Therapeutic discontinuation (switch to 2nd generation TKI)	10 (15.2%) (intolerance/resistance)	2 (9.1%) (intolerance/resistance)
Mortality	4 (6.1%)	1 (4.5%)

CHR: complete hematologic response; CCyR: complete cytogenetic response; MMR: major molecular response; CMR: complete molecular response; TKI: tyrosine kinase inhibitor

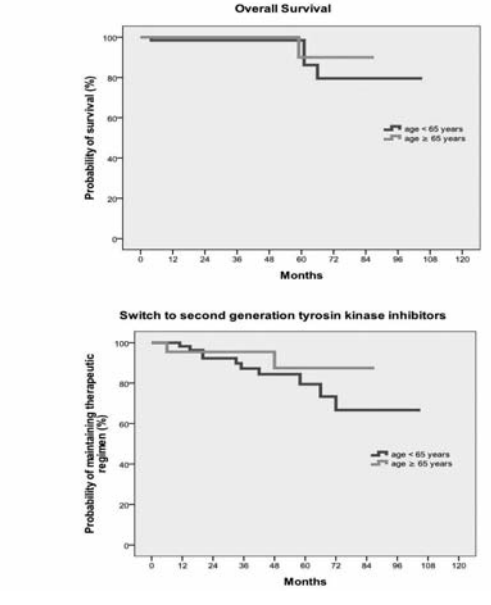

Figure 1.

Results. Figure 1. *Conclusions.* The results suggest that Imatinib treatment is equally effective in young and in elderly patients, having the same toxicity profile. The establishment of tyrosine kinase inhibitors as the standard first line therapy in CML has lessened the negative impact of age on the prognosis of CML.

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MUTATIONS OF THE BCR-ABL- KINASE DOMAIN EXPLAIN THE IMATINIB FAILURE ONLY IN A MINORITY OF PATIENTS WITH CHRONIC MYELOID LEUKEMIA

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Background. The tyrosine kinase inhibitor Imatinib have opened a new era in CML treatment by allowing a targeted disruption of a signalling pathway believed to be crucial for leukemia maintenance. However, soon it was realized that point mutations in ABL kinase domain are responsible for aquired resistance and poor outcome especially for patients with mutations affecting P-loop domain. Clinical impact of imatinib-resistance mutations in CML is still elusive due to controversial reports in literature. *Aims.* our aim was to asses the importance of mutational status for patients with failure or suboptimal response to imatinib. *Methods.* we used a semi-nested PCR for the amplification of the kinase domain of BCR-ABL fusion gene, followed by direct sequencing (Sanger method). This method allows identification of mutations when mutated clone have reached an abundance threshold of ~20-25%. *Results.* from 230 patients in monitoring in our center 35 were referred for mutation detections due to failure or suboptimal response to imatinib. The following mutations were identified: E450K, E459K, F359V, M244V, E255K, L387M, E450A, Q252H in seven patients. Five patients presented single mutation in ABL kinase domain and two patients presented two mutations. Patients with two mutations presented L387M + M244V and respectively, E450A+Q252H. For these seven patients clinical evolution of disease was evaluated: five patients have better clinical evolution after changing treatment to second generation TKIs (nilotinib for two patients and dasatinib for three patients) and two patients died with progressive disease despite changing the therapy to dasatinib. *Summary.* identification of the ABL kinase domain mutations is an important prognostic factor for clinical outcome of patients presenting failure or suboptimal response to imatinib treatment, but presence of these mutations can explain unsatisfactory response only in some patients (seven of thirty-five). For better clinical management, we need new tools able to give us, in real time, a comprehensive information about TKIs efficiency.

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REFRACTORINESS TO IMATINIB - A SINGLE CENTRE EXPERIENCE

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Background. Imatinib (IM) is the first line drug therapy for patients (pts) with chronic myeloid leukemia (CML). While the efficacy of IM is unquestioned, persistence of minimal residual disease and the development of resistance had a significant impact on the clinical management of CML. The mechanisms of resistance have been extensively studied and the search for more efficacious agents to overcome Imatinib failure is still on going 1. *Aim.* To assess the incidence of tyrosine kinase inhibitor (TKI) resistance in CML pts treated in our centre and estimate the mutational rate in this group of pts. *Methods and Results.* 63 pts with a medium follow-up of 46 months (mo) on conventional dose IM therapy were serially monitored by RQ-PCR for BCR-ABL transcripts in peripheral blood samples according to Leukemianet guidelines. Screening of BCR-ABL mutations was performed by direct sequencing covering the entire tyrosine kinase domain (KD). Forty (63%) pts responded to IM (mean follow-up 52 mo). IM was first line therapy in 23/40 (57%). The others received IFN-alfa (36%) or BMT (7%) prior to IM therapy. MMR was obtained after a medium time of 12 mo. Five pts achieved sustained CMR . Twenty three (37%) failed IM therapy after a mean follow-up of 31 mo. Kinase domain (KD) mutations were detected in 7 pts (30%): F317L, E459K, E255K, E255V, M351T, T315I and one pt bearing two mutations L387 (++)/ D276G(+). Five IM resistant pts died, four with disease progression. KD mutations were detected in three pts

after a median follow-up time of 10 mo (range 4-61): F317L, E459K and E255K. Pts bearing F317L and E459K died in myeloid blast crisis and E255K pt in lymphoid blast crisis. The three pts with mutations were treated with Dasatinib but CCyR was never achieved. The remaining 18/23 IM resistant pts received IM during a mean time of 34 mo. None had disease progression and KD mutations were detected in four pts: E255V, M351T, T315I and one pt bearing two mutations L387 (++)/ D276G(+). Fourteen pts (14/23, 22%) with IM failure received Dasatinib therapy for a mean time of 16 mo (range 40-2), of which only two obtained MMR, one with M351T and one with primary resistance and no mutations. Of the Dasatinib refractory pts, one is on HU awaiting BMT and four were switched to Nilotinib. Following a mean time of 11 mo (range 27-16), no major molecular responses were observed in the latter group. *Conclusions.* Careful molecular monitoring according to European Leukemianet Guidelines2 allow early detection of resistance and optimal disease management. The long-term efficacy of second-generation TKIs may also be related to specific BCR-ABL mutations3. We highlight that in our experience only 7/23 pts with IM failure developed KD mutations and that BCR-ABL over expression, drug efflux an influx transporters and BCR-ABL independent mechanisms of resistance may also play a role in IM performance. Choice of therapy should be guided by multiple factors, including mutational analysis, disease phase, patient characteristics, and the safety profile of the agents.

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A CASE REPORT WITH E6A2 BCR-ABL FUSION TRANSCRIPT WITH MARKED BASOPHILIA AND HIGH ACTIVATION OF SRC KINASES DOWNSTREAM BCR-ABL

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Background. Most cases of chronic myeloid leukemia (CML) carry *BCR-ABL* fusion transcripts β2α2 or β3α2 in major BCR region, other fusion transcripts are rare. Until now, *BCR-ABL* fusion transcript e6a2 with breakpoint in intron 6 of the *BCR* gene was detected only in a few CML cases. *Methods.* RT-PCR and sequencing were used to detect the type of *BCR-ABL* transcript. *BCR-ABL* mutational status was assessed by sequencing of the RT-PCR products. The *in vitro* test of sensitivity to TKIs was based on detection of inhibition of phosphorylation of Crkl and Src family kinases (SFK, Tyr416) using immunoblotting. *Features at presentation.* A 51-year-old man presented in January 2010 with 3-week symptoms linked to marked splenomegaly. *Laboratory results.* HGB 7.3 g/dL, WBC 150.3×10^9/L (neutrophils 18%; lymphocytes 2%; eosinophils 18%; basophils 45%; metamyelocytes 4%; myelocytes 5%, promyelocytes 6%, blasts 2%) and PLT 36×10^9/L. Severe anemia and trombopenia were asymptomatic. Cytogenetics revealed 90% of Ph+ cells in the bone marrow (iFISH 278/308 BCR-ABL+ nuclei) with no additional clonal chromosomal abnormalities. Laboratory results implied CML in accelerated phase. Sokal risk was intermediate. Sequencing analysis of amplified BCR-ABL transcript revealed a rare e6a2 fusion, with no evidence for Bcr-Abl kinase domain mutation. Western blot analysis revealed high activation (phosphorylation) of Crkl and the Src family of kinases (P-SFK) in the patients' sample. *In vitro* test of sensitivity of the patients' leukemic cells to imatinib showed sensitivity of Bcr-Abl tyrosine kinase to imatinib (as assessed from P-Crkl monitoring), and a complete elimination of P-SFK, suggesting, that P-SFK represents only Bcr-Abl-dependent SFK activity. *Treatment results.* Cytoreduction was performed with hydroxyurea. Dose of imatinib was 400mg daily. After 2 weeks of imatinib further drop in platelets demanded transfusion of packed platelets. Moreover, G-CSF was indicated for severe neutropenia, while the imatinib dosage was reduced to 400 mg 5 days weekly. At 3 weeks of imatinib therapy (a week after G-CSF) complete resolution of splenomegaly and marked laboratory improvement was observed: HGB 9.6 g/dL, WBC 9.8×10^9/L (eosinophils 1%; basophils 14%), PLT 425×10^9/L. A search for voluntary hematopoietic stem cell donor was started. *Conclusions.* A higher frequency of advanced CML phases has been suggested for the patients with e6a2 rearrangement, but with good initial responses to imatinib. The patient presented here adds additional knowledge to the e6a2 patients: he presented marked splenomegaly, basophilia, severe trombopenia and anemia and a high activation of SFK downstream Bcr-Abl. Further follow-up of the patient should bring novel insights into prognosis of patients with rare e6a2 BCR-ABL transcript variant encoding for Bcr-Abl kinase with possibly enhanced oncogenic potential. *Acknowledgement.* Supported by grants NS9949-3 (Ministry of Health) and MSM 6198959205 (Ministry of Education, Youth and Sports) of the Czech Republic.

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SUCCESSFUL PREGNANCIES IN CHRONIC MYELOID LEUKAEMIA MALE PATIENTS ON IMATINIB THERAPY.

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Imatinib represents the first line management in chronic myeloid leukemia (CML).This therapy has changed the natural history of disease, so that the management of disease must now to adapt to the patient's lifestyle. So, it is not unusual that some patients may hope to have a child. Otherwise, we know that Imatinib therapy has potential teratogenicity in animal, but the effect during conception and pregnancy in human is not clear so far. We report here on 4 healthy pregnancies conceived from four male patients on long-term imatinib therapy. The first patient received CML diagnosis in 1990 at the age of 27y. After interferon therapy, he underwent to stem cell auto transplantation in 1998; in march 2000 he started imatinib at full dose. In October 2006 the patient showed BP of CML and he switched to Dasatinib. Then, for not compliance to therapy, he switched to Nilotinib until July 2008, when he showed a new BP of CML and he died in September 2008. During Imatinib therapy the patient underwent to sperm criopreservation; in 2007 the wife began pregnant and in 2008 and she delivered three healthy babies. The other patient, after a CML diagnosis in May 2002, when he was 34 yo, started imatinib in May 2003. In May 2005 he showed AP of CML and during this period the partner began pregnant and after 34 weeks she delivered an healthy baby. The patient switched to Dasatinib in July 2005 and he is already treated with that drug in complete cytogenetic and major molecular response. The third patient had CML diagnosis in February 2006 at age of 39y, starting Imatinib therapy in march 2006. The patient's partner had an history of recurrent spontaneous abortions, but she began pregnant in 2008 while the partner was receiving Imatinib. They had an healthy baby after 34 weeks of pregnancy. The last patient received CML diagnosis in March 2004 when he was 26yo, starting Imatinib at full dose. This treatment led to complete cytogenetic response after one year with major molecular response in 24 months. The patient's partner conceived in 2009 during Imatinib treatment. After 34 weeks, she delivered an healthy baby. In this report all patients were on prolonged Imatinib treatment. In all the babies were not observed structural malformations and they all have normal growth and development until now. There is an increasing evidence that children born to men who were taking Imatinib at time of conception do not have an increased risk of congenital malformations or increased risk of pregnancy associated complications. It is useful anyway to be advised patients in Imatinib therapy to practice adequate contraception, even if for male patients fathering children can be achieved without interruption of treatment.

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PRIOR THERAPY WITH INTERFERON IMPROVES THE RESULTS OF SECOND-LINE TREATMENT OF CHRONIC MYELOID LEUKEMIA WITH IMATINIB

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Background. Treatment of chronic myeloid leukemia (CML) with imatinib mesylate (IM) administered frontline covers only about 50% of all patients in Ukraine due to certain reasons. Therefore the issue of adequate treatment of these patients for the time they are on a waiting list for the drug, which may last from 6 to 12 months, is of current importance in our country. *Methods.* The group of 77 patients with CML treated with imatinib for 2-4 years was analyzed for treatment efficacy. Among these patients 16 took the drug frontline (group 1); 35 patients were treated with imatinib after prior therapy with interferon alfa (group 2); another 26 patients were pretreated with hydroxyurea and in more rare cases - with busulfan (group 3). The dose of imatinib was 400-800 mg daily. *Results.* In the first group of patients major cytogenetic response (MCyR) was achieved in 64% of patients at 6 months and in 68% of patients at 12 months. Two of these patients (12%) developed blast crisis in 3 and 4 months after imatinib introduction. Both of them were qualified as high risk group, their daily dose of imatinib was 600 mg. All the other patients developed complete cytogenetic response

(CCyR) at 24 months. In the second group of patients mean duration of interferon alfa treatment prescribed for the chronic phase of CML was 21,2 months before imatinib initiation. Similarly to the first group MCyR was achieved in 62% of patients at 6 months and in 71% - at 12 months. Blast crisis was diagnosed in 5 patients (14.3%). Complete CyR was achieved and remained stable in 66% of patients at 24 months. Among the patients of the third group MCyR was achieved only in 15 % of patients at 6 months and minor cytogenetic response - in 35% of patients at the same time point. Rest of the patients in this group had no cytogenetic response at all. The proportion of patients expressing major cytogenetic response increased to 19% at 12 months and subsequently to 23% - at 24 months. Blast crisis was diagnosed in 7 of 26 patients (27%); 5 of them were pretreated with busulfan. *Conclusions.* The results of this study show that the level of cytogenetic response and rate of transformation to the blast crisis were similar in patients taking imatinib frontline and in those who took the drug as second-line treatment after prior therapy with interferon alfa. Significantly worse results were revealed in patients pretreated with hydroxyurea and busulfan for the long period of time. One may conclude that in treatment naïve patients and in patients pretreated with interferon there may remain preserved a pool of Ph-negative hemopoietic cells able to replace the leukemic clone in bone marrow after treatment with imatinib. Therefore in case there is no possibility to administer imatinib frontline due to various reasons, initial treatment with interferon alfa is probably an optimal option for patients with CML as pretreatment with other cytostatic drugs can significantly reduce the response rate for subsequent treatment with imatinib.

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RESULTS OF CHRONIC MYELOID LEUKEMIA TREATMENT BY IMATINIB IN UKRAINE

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Background. Up-to-date therapy of chronic phase of chronic myeloid leukemia (CML) is based on using of tyrosine kinase inhibitors. The application of imatinib for treatment of CML in Ukraine have been actively started since 2008. *Aims.* To estimate the efficiency of imatinib application in CML patients after 12 months of treatment . Here, we present a restricted analysis of Ukraine cohort of CML patients. *Methods.* In research we included 558 patients with the chronic phase CML in age from 18 to 80 years old. Clinical data were collected at most recent visit and retrospectively through clinical chart review and Sokal Score. Diagnostics and monitoring of CML was conducted on the basis of cytogenetic examination of bone marrow cells by G - banding, and also molecular-genetic research of bone marrow and peripheral blood cells by the RT - PCR. 35% patients have got imatinib as the first line therapy and 65% patients have taken other medicinal preparations before (hydroxyurea, busulfan, interferons). Duration of prior treatment was 4-120 months. *Results.* Results of CML patients monitoring were estimated after 12 months of imatinib treatment according to recommendations of ELNet 2010. A complete cytogenetic response was got for 35% patients, partial cytogenetic response with level of Ph-chromosome <35% was revealed in 25% patients. 40% patients had more than 35% Ph-positive cells in bone marrow after 1 year of imatinib taking. The hematological toxicity during the imatinib reception developed as anaemia (Hb<80 g/L) in 3% patients, leukocytopenia ($<2\times10^9$/L) - 4% patients, thrombocytopenia ($<50\times10^9$/L) - in 7% patients. 2% patients discontinued imatinib during the follow-up period because of hematological and unhematological toxicity of preparation. For patients who have got imatinib as the first line therapy, frequency of complete cytogenetic response was significantly higher, than for the pre-treated patients (P<0.05). The least level of complete cytogenetic response was among patients getting busulfan before in comparison with patients who were pre-treated by other preparations (P<0,05). *Conclusions.* Treatment of chronic phase CML by imatinib is the most effective as the first line therapy in comparison with pre-treated patients, especially patients, who have taken busulfan.

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SEQUENTIAL CHRONIC MYELOGENOUS LEUKEMIA (CML) AND CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) - 3 CASE REPORTS

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Background. Incidence of second malignancies in CLL is well-known. Coincidence of CLL and CML is a rare event. We report three patients in whom CLL and CML were diagnosed sequentially. The possibility that both diseases may arise from the same clone may have important implications for pathogenesis and clinical management. *Results.* CASE 1. A 57-year-old man was diagnosed with B-CLL by immunophenotyping indicated due to non-symptomatic lymphocytosis in October 2007. Clinical stage was Binet A, karyotype +12, IgVH gene unmutated. Due to short lymphocyte doubling time one cycle of fludarabine and cyclophosphamide was given. After recovery leukocytosis with immature granulocytes was observed and Ph-positive CML with $\alpha2\beta3$ BCR-ABL was diagnosed in August 2008 (Sokal risk intermediate). Patient started treatment with imatinib 400 mg daily in September 2008 and achieved complete cytogenetic response (CR) in May 2009 and major molecular response (MR) in January 2010. Curiously a third malignancy - lung adenocarcinoma (clinical stage IV, T1N3M1 with spread to mediastinal lymph nodes and blade bone) was diagnosed in June 2008. While imatinib treatment was ongoing, gemcitabine was added. The patient is currently alive in satisfactory condition. CASE 2. A 57-year-old man was diagnosed with Ph-positive $\alpha2\beta3$ BCR-ABL CML in July 2009 due to non-symptomatic leukocytosis (Sokal high risk). He started therapy with imatinib 400 mg daily in August 2009 and achieved major CR in January 2010. In February 2010 absolute lymphocytosis and thrombopenia were observed. Immunophenotyping confirmed B-CLL; ZAP 70 was negative, IgVH gene unmutated. Clinical stage was Binet A and no treatment for CLL was indicated. However, acceleration of CML with 30 % of basophiles was observed the same month. Patient was converted to dasatinib and the possibility of allogeneic stem cell transplantation has been discussed. CASE 3. A 71-year-old man was diagnosed with Ph-positive $\alpha2\beta2$ BCR-ABL CML in December 1999. He was treated with interferon alfa and oral AraC. No CR was achieved and because interferon toxicity, patient was assigned to imatinib 400 mg per day in July 2001. Complete CR was achieved in January 2006, major MR was not achieved. Due to absolute lymphocytosis immunophenotyping was indicated and B-CLL (clinical stage Binet A, karyotype with deletion 13q14, IgVH gene mutated) confirmed in September 2004. No therapy was required until February 2009, when chlorambucil and prednisone was given for anemia and short lymphocyte doubling time. Imatinib was interrupted during CLL therapy and cytogenetic progression was observed. Despite reintroduction of imatinib hematologic progression with thrombocytemia and F311L mutation of tyrosine kinase domain developed and patient is now assigned to 2nd generation tyrosine kinase inhibitor. *Conclusions.* In neither of abovementioned cases common origin of CLL and CML was demonstrated. Sequential occurrence of CML and CLL due to the prolongation of patients´ survival might be a more frequent event in the future. Clinical management of these patients may be challenging. *Acknowledgement.* This work was supported by the grants NS9949-3 (Ministry of Health) and MSM 6198959205 (Ministry of Education, Youth and Sports) of the Czech Republic.

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DASATINIB IS A POTENT AGENT IN SECOND LINE TREATMENT FOR CHRONIC PHASE CHRONIC MYELOID LEUKEMIA PATIENTS - SINGLE CENTER EXPERIENCE

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Background. Dasatinib 100 mg once daily is approved for chronic-phase chronic myeloid leukemia patients resistant or intolerant to Imatinib. According to European Leukemia Net recommendations failure to Imatinib first-line in early chronic phase is defined as less than complete cytogenetic response (CCyR) after 12 months of treatment or loss of CCyR at any time during treatment. We tried Dasatinib efficacy with a 6 months minimum follow-up in 16 patients resistant to Imatinib.

Patients. All 82 patients of our center with Philadelphia chromosome (Ph) - positive chronic myeloid leukemia in first chronic phase were initially treated with Imatinib in standard dose 400 mg once daily. In the first group of 12 patients of this front-line treatment failed in one-year observation - they did not achieve any cytogenetic response. In one case E450Q mutation was detected and reported to be sensitive to Dasatinib. The second group consisted of 4 patients who lost CCyR in a median period of 40 months since the start of Imatinib. No mutations were detected in this group. All 16 patients were switched to Dasatinib 100 mg once daily. *Results.* Among these 16 patients observed for at least 6 months, 11 patients (69%) achieved CCyR and 8 patients (50%) achieved a major molecular response (MMR). There were differences in response rate by groups. In the first group of 12 patients primary resistant to Imatinib CCyR and MMR were achieved by 7 (58%) and 4 (33%) patients respectively. All patients in the second group achieved CCyR and MMR by 6 months. Grade 3 or higher neutropenia and thrombocytopenia occurred in 31% and 25% of patients respectively. Nonhematologic toxicity was grade 1 or 2. All patients are alive and all Dasatinib responders are still maintaining CCyR. 3 of 5 patients who failed Dasatinib therapy were eligible for allogeneic bone marrow transplantation. *Conclusions.* In our center experience Dasatinib as a second line therapy for first chronic phase myeloid leukemia patients resistant to Imatinib produces high rates of CCyR and MMR with an acceptable toxicity profile.

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TRABECULAR BONE DENSITY INCREMENT IN CML PATIENTS UNDERGOING IMATINIB THERAPY

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Background. Several studies have demonstrated that Imatinib, a tyrosine kinases inhibitor, alters calcium and phosphate metabolism. 1. We evaluated if these alterations are related to a bone-metabolic disbalance. 2. We propose a possible explanation for the abnormalities in bone and mineral metabolism. *Methods.* Transversal analysis of CML patients treated with Imatinib from April 2006 to february 2010: 18 patients, 8 male (44.4%) and 10 female (55.6%), with a median age of 56 years (25-83), in treatment with a median daily Imatinib dose of 511 mg (400-800), for a median period of 41 months (1-83). Twenty one patients with myeloproliferative sindromes Philadelphia chromosome negative served as internal controls: 10 male (47,6%) and 11 female (52,4%), with a median age of 54 years (28-80). We evaluated bone mineral density (BMD) by fotonic dual absorciometry at the beginning of the study, 24 and 48 months later. Trabecular bone volume (TBV) was determined in patients and controls by microscopic study of bone marrow biopsies with an ocular-mounted Weibel II graticule (40x). We calculated the bone percentage of all bone marrow. *Results.* BMD was higher in 29 of the 44 determined (66%), although the median values din´t differ significantly between the first determination, second (two years later, P=1) and third (four years later, P=1). However, the percentage of TBV after Imatinib therapy was significantly higher than this for the control group (31.5 *vs.* 15.3 % P=0.002). *Conclusions.* 1. Trabecular bone volume is increased in patients receiving Imatinib. 2. This alteration could appear soon in early Imatinib therapy and stabilce through the treatment, since BMD didn´t change after two years of monitoring. 3. Imatinib could interfere with osteoblasts and osteoclasts functions by inhibiting alpha and beta PDGF receptors.

No Conflict of Interest. Informed consent was obtained.

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MEGAKARYOCYTIC MATURATION IN PERIPHERAL BLOOD SMEAR IN CHRONIC MYELOID LEUKEMIA

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Background. Knowledge about how megakaryocytes mature and platelets form, has come chiefly from direct observations on bone marrow, or from studies on megakaryocytic cells grown in culture, and less from direct observations on the peripheral blood (PB). Circulating megakaryocytic cells, abundant in all phases of chronic myeloid leukemia (CML) in India, seem, however, to point to an alternative viewpoint. *Aims.* To evaluate how megakaryocytes mature and platelets form in the PB in CML. *Methods.* Romanowsky-stained PB of 1200 patients of CML in the 3 phases of the disease was evaluated to assess the type of morphologically identifiable Mk cells. Immunocytochemistry (APAAP) for megakaryocytes was done in selected cases. *Results.* The most dramatic features are seen in the blastic and accelerated phases. There is a continuum of megakaryocytic maturation that begins with a megakaryoblast differentiating abnormally, and ends with a mature, functioning micromegakaryocyte. Blasts otherwise undifferentiated, reveal their lineage unequivocally by putting out into the circulation, dysplastic platelets / megakaryocytic cytoplasmic fragments that arise from a localized zone of the cell membrane. While the periphery continues to be engaged in abortive and tentative attempts at successful platelet formation, cytoplasmic granules appear, heralding shift towards normal maturation. The process culminates in a small mature micromegakaryocyte, which continues to form normal platelets. *Conclusions.* 1. Megakaryoblasts are capable of platelet production without undergoing normal maturation. This unproductive effort, that yields dysplastic platelets, corrects itself as the cell matures further. 2. The familiar mature micromegakaryocyte is not a dyspoietic cell but one that actively contributes to platelet formation. 3. Large dysplastic platelet masses in the PB in CML arise largely from the PB megakaryocytic cells. 4. Study of PB Mk cells allows one to see megakaryocytic cells in a new perspective and opens vistas for further study.

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PSYCHOLOGICAL PROFILE OF CHRONIC MYELOGENOUS LEUKEMIA (CML) PATIENTS

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Background. The studies of the psychological peculiarities of personality of CML patients are absent. *Aims.* 1. To study conceptual and dynamic peculiarities of mental activity (temperament structure). 2. To study individual and typological peculiarities of a personality. 3. To determine dominant emotional state during the treatment process. 4. To study peculiarities of axiological and conative spheres. *Methods.* 25 CP CML pts (12 men and 13 women) on TKI treatment were studied. "Questionnaire of temperament structure"(Rusalov V. 2007), "Individual and typological questionnaire"(Sobchik L. 2008), "Method of dominant state determination"(DS) (Kulikov L. 2002), "Life orientations"(LO) (Crumbaugh G., Maholick L., 1981; Leontyev D. 2000) were used. This complex of methods is aimed to study psychological profile in interrelations of different levels and substructures of personality-biologically determined, constitutional, individual-typological, emotional-affective, volitional as well as value-motivational peculiaraties formed during socialization. Healthy people were used as controls. *Results and discussion.* Temperament study revealed differences (P<0.01) in the "rate of psychomotor action"parameter. CML pts are closer to low emotional and passive type (using classical terminology - close to phlegmatic type). It seems that feature of weakness of ego is the main in the personality, whereas overestimation is compensatory. It was shown that accentuated personality traits of CML patients are: spontaneity (increased self-esteem and striving to lead), constriction (inflexibility, inability to change the line of behaviour following the change of real-life situation), vulnerability (sensitivity and anxiety). Such combination of accentuated personality traits is disharmonious and creates obstacles for social and psychological adaptation. Dominant emotional state is characterized by the following parameters: "Passive attitude to life situation"(P<0.001), "Calmness" (P<0.05), "Satisfaction with life" (P<0.01). The obtained result reflects the patients' compensative striving to show externally that they are successful, satisfied with their self-realization, able to take over the responsibility, make their choice and overcome difficulties while implementing it. In axiological and conative studies there were differences in regard to the following scales: "Life purpose" (P<0.001), "Life productivity or satisfaction with self-actualization" (P<0.05) and "I am the locus of control" (P<0.05). In each of these cases the patients' indicators exceeded normal data. This proves that self-consciousness of patients with CML reflects the aims, which make their life meaningful and fill temporary prospects. At the same time the plans that are formed do not have a real ground in the present and are not supported by personal responsibility for their realization. *Conclusions.* In terms of their temperament characteristics CML pts have on average the low emotional and passive type. There is a disharmonious combination of accentuated features of spontaneity, rigidity, vulnerability in patients with CML. A combination of passive attitude to life, satisfaction and reduced level of concern with the actual life situations can be seen in the emotion-

al structure of the patients. In axiological and conative spheres of patients with CML prevails satisfaction with the results of life. The data obtained can be used for psychological support of the therapeutic process.

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CHRONIC MYELOID LEUKEMIA TREATMENT IN BLASTIC PHASE WITH IMATINIB 600 MG

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Background. Management of Blast crisis Chronic Myeloid Leukemia (BC-CML) is the most challenging entity in the treatment of chronic myeloproliferative disorders. In 2000, the introduction of Imatinib mesylate (IM) has opened a new option in the treatment of BC-CML. Early results have shown the superiority of IM compared to conventional chemotherapy. *Aims.* To investigate the long-term effects of IM in BC-CML patients, treated with IM 600 mg daily. *Methods.* Twenty-five patients were enrolled. After a 3 year follow-up, we assessed the IM long-term efficacy, response duration and survival, and we characterized the prognostic factors associated with a favorable outcome. Patients were monitored for hematologic and cytogenetic response at 1-3 months intervals. A complete hematologic response (CHR) required the normalization of platelet and white cell differential count and absence of extramedullary involvement. The definition of return to chronic phase (RTC) required less than 15% blasts and less than 30% blasts plus promyelocytes in blood or bone marrow and less than 20% peripheral basophils. Cytogenetic analysis was performed with standard banding techniques and the response was rated as usual. *Results.* Thirteen patients had a sustained RTC, and 8 patients achieved a CHR. RTC was subsequently lost by 7 patients for a median duration of a second CP of 9 months (range 1-32). Four patients lost the CHR, for a median duration of the CHR of 6 months (range 1-23). Six patients had a cytogenetic response (2 complete, 1 partial, and 3 minor or minimal). The Kaplan-Meier median survival time was 6 months, and the survival rated were 43% at 6 months, 19% at 12 months, 13% at 18 months and 15% at 36 months. Conclusion. We confirm that IM as monotherapy was valuable and safe in the short-term, but relapse rate was high and the longer term clinical outcome was not significantly influenced.

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CHRONIC MYELOID LEUKEMIA (CML) TREATMENT EXPERIENCE AT HOSPITAL J.M. RAMOS MEJIA (HRM). A DESCRIPTIVE STUDY FROM ARGENTINA

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Background. The rate of complete cytogenetic response (CCyR) in patients with chronic phase CML treated with imatinib mesylate is 87%. Data in our country is limmited. *Aims.* to describe demographic, clinical presentation and treatment characteristics and outcome of CML patients treated at HRM. *Methods.* data of patients with CML diagnosis since 2000 was collected from medical charts: demographic characteristics, clinical presentation, type of treatment and outcome. The rate of cytogenetic response to tyrosin kynase inhibitors (TKIs) at 6, 12 and 24 months of patients treated with imatinib mesylate and second generation TKIs(2° TKI), nilotinib and dasatinib, as initial treatment is reported. *Results.* Data of 96 patients was analysed with a median follow-up of 35 months (IQR: 13-59). Median age was 42 years (range 5-79) and male sex 52% (50/96). Sokal risk score was calculated in 86% of patients (83/96) with 19% (16/83) low risk, 28% (23/83) intermediate risk and 53%(44/83) in the high risk group. 93% (87/96) of patients were diagnosed in chronic phase, 2% (2/96) in accelerated phase and 5% (5/5) in blast crisis. 26% (25/96) of patients had received Interferon prior to TKI treatment. First line TKI was imatinib in 75%(n=72) of patients, nilotinib in 4%(n=4), dasatinib in 10%(n=10) and 2 (2%) received allogeneic bone marrow transplantation. 8 patients (8%) were lost to follow-up. Data of patients treated with imatinib and with dasatinib is described in Table 1. 29% (25/86) of the patients required second generation TKIs, 28%(24/86) because of failure to achieve response and 6%(5/86) for intolerance. Mortality rate was 14%, in 10 cases related to CML and in 4 patients not related. *Summary.* A cohort of patients treated in a Public Hospital in Argentina is described. Results show 53% rate of complete cytogenetic response in CML chronic phase patients treated with first line imatinib. More studies are necessary to confirm this outcome and to identify the variables that have influence on these results.

Table 1.

Table 1	Age	Male (%)	SOKAL RISK SCORE (%)			% CCyR			Mortality (%)
			1	2	3	6 months	12 months	24 months	
Imatinib	41	50(36/72)	19(14/72)	31(23/72)	34(25/72)	31(17/55)	47(27/57)	53(31/59)	8(13/72)
2ª TKI	45	50(7/14)	0	0	100(14/14)	71(10/14)	79(11/14)	NA	14(2/14)

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BUSULFAN, AND NOT DURATION OF DISEASE, RESULTS IN DECREASE OF RESPONSE TO IMATINIB IN VERY LATE CHRONIC PHASE OF CHRONIC MYELOID LEUKEMIA (CML)

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Background. Imatinib (IM) is very effective in CML in its early chronic phase (CP) whereas the results of therapy are much lower in patients in late CP. Few data are available about outcome of patients in very late CML CP (disease duration ≥5 years) and pretreated by busulfan. *Aims.* The aim of this study was to analyze the efficacy of Glivec in busulfan pretreated CML CP patients with long history of the disease. *Patients and methods.* In our database there are 14 patients with very late CML CP treated by busulfan before IM. The efficacy of IM in this group was compared to 19 pts with very late CML CP, who have never been treated with busulfan. At the start of Glivec the median age for pts treated with or without busulfan was 58.7 yrs (range 24-84) and 51 yrs (range 41-78.5). The median time before Imatinib was 90 mos (range 64-243) and 76 mos (range 61-122 mos) for busulfan and non-busulfan group respectively. Median time of Imatinib therapy was 33 mos (range 5-72 mos) and 35.5 (range 2-83mos) in pts pretreated or did not pretreated by busulfan respectively. There was another control group of pts with disease duration less than 60 mos before Imatinib (early late CP). *Results.* The rate of CCyR was 64% (41/64) in early late CP, 50% (8/16) in non-busulfan group and only 14% (2/14) in busulfan group of pts. Probability of 5-years of overall survival (excluding CML unrelated death) was 88%, 83.5% and 68% for pts in early late CP, with or without pretreatment with busulfan. The rate of CML related death was higher in pts pretreated with busulfan (27%) than other groups - 9% and 16% in early late CP and busulfan naïve pts in very late CP group. Glivec related toxicity led to dose interruption or treatment discontinuation in 28% (4/14) and 21% (4/19) pts with or without busulfan therapy (p>0,05). Some of busulfan pretreated patints resistant to imatinib responded to another TKIs. *Conclusions.* The rate of CCyR and overall survival were comparable in pts with early late CP and very late CP pts without experience of busulfan therapy. Thus, long-term duration before imatinib treatment does not result in decrease of sensitivity to Imatinib and in contrast to widely accepted opinion about accumulation of mutations leading to insensitivity to imatinib Busulfan pretreatment results in decrease of sensitivity to imatinib, although not spread to another TKIs.

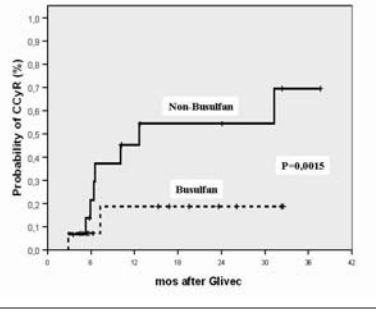

Figure. CCyR on IM in pts pretreated or not with busulfan.

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COMPLETE CYTOGENETIC RESPONSE ON LOW DOSE CHEMOTHERAPY AND 2ND GENERATION TKI NILOTINIB IN A MULTI-TREATED CML PATIENT ONE YEAR AFTER LYMPHOID BLAST CRISIS

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Background. Chronic myeloid leukaemia (CML) is nowadays a highly curable disease in its chronic phase (CP), and sustained complete response (CR) - most often cytogenetic (CCyR) - can be obtained with first-line imatinib treatment. CML blast crisis (BC) is fatal within few months unless a second CR is achieved (most often with acute leukaemia regimens) and bone marrow transplantation (BMT) can be performed. Elderly, unfit patients do not usually benefit of this kind of approach. Tyrosin-kinase inhibitors (TKI) can safely be administered to the vast majority of patients, and have been proved useful in resistant cases in the chronic and accelerated phase. Mutational analysis can drive the choice of 2nd generation TKI when a first line of treatment with imatinib is no longer effective. *Aim.* to describe the case of Mr S., a 72-year-old man suffering from CML, who suddenly developed lymphoid BC, B-cell type, while on dasatinib. *Case report.* Mr S. was 60 when he was diagnosed with CML-CP. Caryotype showed t(9; 22). Sokal score was high. Older age and the first signs of chronic obstructive pulmonary disease made him unfit for BMT. After a few months of interferon and Ara-C, he was switched to hydroxyurea because of unacceptable toxicity (namely, psychiatric). In 2002, loss of hematologic response and availability of the first TKI resulted in a therapeutic change (imatinib 400 mg/day) leading to CCyR 6 months later. Imatinib was continued at 400 mg until 4 years later, when cytogenetic relapse was documented, accompanied by t (12;15) additional mutation. 6 month later, despite dose-increase and adequate imatinib plasma levels, no response was obtained. Mutational analysis showed bcr-abl H396L mutation. Dasatinib 140 mg/d was then started, and Mr S. could again, 6 months later, obtain CCyR. Response was short-lived, and 10 months after dasatinib was started, lymphoid BC, B-cell lineage was documented in the peripheral blood (PB) and the bone marrow (BM blasts >90%), harbouring a complex caryotype and a new mutation (V299L) in addition to the known cytogenetic and molecular abnormalities. High dose steroids and weekly vincristine led to PB blast clearance by day 8, but no effect was found on BM. At day 21, Mr S. wished to go back to his native country, and he left with a palliative treatment (oral 6-mercaptopurine 50 mg/day, oral prednisone 100 mg/day, IM Methotrexate 25 mg/week). He unexpectedly came back 8 months later, and both PB and BM examination confirmed morphological CR of lymphoid BC. Cytogenetic study of BM showed major cytogenetic response (2/22 metaphases were positive for t(9;22)). Nilotinib was started at 800 mg/day. Complete cytogenetic response was obtained one month later and remission was confirmed 3 months afterwards. *Conclusion.* low dose chemotherapy and nilotinib have been effective in obtaining cytogenetic remission one year after lymphoid blast crisis on dasatinib in an elderly patient not fit for BMT. Hypothetically, associating low-dose chemotherapy and 2nd generation TKI could be more effective than using either treatment alone. Mutational analysis could be used in this context to drive TKI choice.

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A RETROSPECTIVE ANALYSIS OF IMATINIB MESYLATE RESPONSES IN CHRONIC MYELOID LEUKEMIA PATIENTS IN THE DEVELOPING WORLD

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The introduction of imatinib mesylate made a remarkable contribution to the management of patients with CML in chronic phase and this drug has now become the standart treatment for adult patients throughout the world. However, access to STI571 treatment became available in a delayed manner in many parts of the world. We assessed the long-term efficacy of imatinib exposure in patients with chronic myeloid leukemia in chronic phase according to European recommendations. Approximately half of the patients (43.6%) had prior history of interferon therapy. The median time from diagnosis to imatinib therapy was 7 months (range, 0-132 months). Of the 94 patients complete cytogenetic responses were achieved in 39.4% of patients; 21.3% had cytogenetic failure and 5.3% without any hematological response. Treatment was tolerated well and 54% of patients with a median follow-up of 60 months (range, 3-168 months) continued to receive imatinib at the intended dose. This study demonstrated that imatinib treatment in chronic phase CML patients is highly efficacious even if applied as an second-line option.

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CHRONIC MYELOID LEUKEMIA IN REUNION ISLAND

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Background. The Epidemiology of Chronic Myeloid Leukemia (CML) in Reunion has not been studied to date. *Aims.* The purpose of this study is to assess the epidemiological and therapeutical aspects of the disease in Reunion, an island where the population has the following characteristics: mixed racial origins: Europe, Asia, Africa, Madagascar (INSEE 2008 data), two main age-groups: young and ageing. *Methods.* This is a retrospective study based on computer data originating from the hematology laboratories of the two centers of the regional hospital complex: Saint-Pierre and Saint-Denis, where all CML patients are supposed to be managed. The time span of the study is 10 years : 01/01/1998 - 12/31/2008 (n=65). Criteria for diagnosis have been the presence of the Philadelphia (Ph) chromosome and/or BCR-ABL transcripts. *Results.* Epidemiological aspects: crude annual incidence is 0.78 per 100,000 (1-2 in mainland France). The average age at diagnosis is 51 (54). The male to female ratio is 0.9 (1.4). At diagnosis, 97% of patients are in chronic phase, 3% are in accelerated phase and 0% are in blast crisis. The Sokal score at diagnosis is low (<0.8) for 35% of the patients, high (>1.2) for 27% and intermediate (0.8-1.2) for 39%. Most of the patients (94%) are Ph positive and 18% of them have related cytogenetic abnormalities. *Therapeutical aspects.* In Reunion, there is a national healthcare insurance system who can cover for medicine and hospitalization. Prior to the introduction of IMATINIB (IM) in Reunion (2002), most of the patients (71%), were treated with INF±Aracytine. Since 2002, 86% have received IM. Among these patients, 61% (n=23) received IM as first-line treatment, 34% (n=13) as second-line and 5% (n=2) as third or fourth line treatment. Causes for therapeutical change under IM are as follows: accelerated phase (n=12), blast crisis on myeloid (n=2) or lymphoid mode (n=1), intolerance (n=5): hematological (n=3), hepatic or other (n=2). Three BCR-ABL mutations have been observed (T315I, G250E, L248V). Eight patients received DASATINIB. One patient received NILOTINIB. Three patients received allogenic transplants (one of whom from placental blood). Two IM-treated patients developed other forms of cancer (urothelial, digestive). At 18 months, the estimated rate of freedom from progression to accelerated-phase or blast crisis was 90% (96.7% in IRIS trial). *Conclusion.* Our study shows that it seems that there are epidemiological specificities to CML in Reunion as compared to mainland France, in particular it seems that it is less frequent and that it affects women more than men. However, since it seems to show the same initial clinical presentation and therapeutic response as it does in metropolitan France "in particular in the case of tyrosine kinase inhibitors" we feel encouraged to suggest that our patients take part in the available clinical trials.

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IMATINIB MESYLATE INDUCED HEPATOTOXICITY IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA (CML)

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Background. Imatinib Mesylate (IM) is current standard treatment for newely diagnosed patients (pts) with CML.Treatment with IM is generally well tolerated, and the risk for severe adverse events is low. Liver toxicity has been observed in 1-5% of CML pts treated with IM in various reports, but in general, liver dysfunction resolves ether with

dose reduction or IM discontinuation. *Aim.* The aim of our study was to analyse pts who developed liver toxicity during IM treatment, from the large cohort of our CML pts. Patient: Between 2002 and 2009, 90 pts with CML were treated with IM in our institution. In five pts liver toxicity occured between 4 to 40 months after IM treatment was started. Pts were closely followed with laboratory monitoring once weekly and later on every 2 weeks. *Results.* We analysed 2 males and 3 females with mean age of 46 years (range, 35-59 yrs). All pts had chronic phase CML, 2 pts had low risk Sokal score, one pt intermediate and 2 pts high risk. Before the introduction of IM treatment all pts had normal values of liver enzymes, normal liver function and were seronegative for hepatotropic viruses HCV and HBsAg. IM therapy was commenced in a dose of 400 mg/d. Liver function tests performed during the first three months of treatment were normal. Liver toxicity developed in our patients after 4-40 months of IM treatment (median 14). Aminotransferases (AST and ALT) values increased up to CTC Grade 2 in 3 pts and CTC Grade 3 in 2 pts. There were no signs of cholestasis and hepatic synthesis was not affected. In one pt, whom the manifestation of Grade 3 hepatoxicity occurred after 12 months of IM treatment, HBsAg was detected, with negative HBeAg but with anti-HBe antibodies present, suggesting reactivation of unapparent HBV.The pt was treated with lamivudine and later on with interferon alpha. In all other pts, IM treatment was withheld until grade 1 toxicity was reached and then restarted. In one pt IM treatment was discontinued definitively due to recidivant toxicity, so the pts continued treatment with nilotinib..*Conclusion.* IM have many adverse events but the incidence and management of hepatotoxicity has been rarely reported. Liver function tests should be carefully monitor during treatment and if they continue to be elevated, treatment have to be discontinued and reassessment for viral reactivation should be done. Continuation of IM in pts with viral reactivation is doubtful and possess a risk, so, introduction of interferon alpha as maintenance therapy could be of great help in preserving good cytogenetic response already achieved with IM.

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EARLY DEVELOPMENT OF SECONDARY HYPERPARATIROIDISM AFTER STARTING IMATINIB THERAPY IN MCL

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Background. The tyrosine kinase inhibitor Imatinib Mesylate has an established role in the management of chronic myeloid leukemia by inhibition Bcr/Abl fussion protein, but some other tyrosine kinases may also be inhibited: PDGFR and C-Kit. Several studies, included ours, have demonstrated that Imatinib theraphy causes anomalies in calcium and phosphate metabolism, such as hypocalcemia, hypophosphatemia and hyperparatiroidism. *Objetive.* To specify in which moment of the treatment the mentioned alterations appear. *Methods.* Prospective study of CML patients treated with Imatinib from July 2006 to June 2009: 9 patients, 5 male (55,6%) and 4 female (44,4%), with a median age of 62 years (29-83), in treatment with a daily Imatinib dose of 400 mg. Serum levels of calcium, phosphate and parathyroid hormone were determined before the beginning of Imatinib treatment, and then monthly until observing a significant difference with regard to the initial determinations. The statistical analysis of the results was performed by Wilcoxon test. *Results.* Significant differences were detected for all the parameters measured after one month of treatment with Imatinib: calcium (8,76 *vs.* 9,52 mg/dL; P= 0,008), phosphate (2,84 *vs.* 3,87 mg/dL; P=0,011) and parathyroid hormone (114,38 *vs.* 56,89 pg/mL; P= 0,008). *Discussion.* Patients taking Imatinib develop an increase in bone matrix mineralization, secondary to a decrease in calcium mobilization from bone (probably by PDGFR inhibition and the consequent aboliton of osteoclast activation). It results in hypocalcemia, hypophosphatemia and in a long lasting secondary hyperparatiroidism. This work is carried out in order to determine when they appear. We note that these analytical changes turn up as soon as after one moth of treatment *Conclusion.* Disorders of calcium and phosphate metabolism appear early after starting Imatinib therapy.

Informed consent was obtained.

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THE IMPORTANCE OF T315I, T317L, E255K AND Y253H BCR-ABL GENE MUTATIONS IN THE PATIENTS OF CHRONIC MYELOID LEUKEMIA WHO TREATED BY IMATINIB

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Aims. In spite of Imatinib is an effective treatment for CML, the important problem that is seen by at the patients is primary and secondary resistance. *Methods.* In this study, the 54 chronic phase CML patients observed in two centre between 2000-2008, mostly observed T315I, F317L, Y253H gene and E255K mutations in BCR-ABL gene are scanned by ASO-PCR method. *Results.* Twenty of patients are men and 42 of patients are women and the median age of the patients are 44.5(19-78). According to the Sokol classification in diagnosis; 12 of patients are low risky, 26 of them are middle risky and 16 of them are in high risky group. Median imatinib usage time is 1.8(0.3-7) year. When imatinib treatment response is evaluated; the 24 of the patients are optimal response, 10 of the patients are suboptimal response and 20 of them have resistance. Expected values in 7 years are found as 96% for Median Overall Survival(OS) and 80% for Progression Free Survival(PFS). Scanned mutations are observed in 18 of the patients and T315I and F317L are observed in 2 patients together. Mutations are determined in 60% of the resistance patients(P=0.004). Seven years PFS is found as 62% in mutation determined patients and 90% in undetermined patients(P=0.041). While T3151 mutation observed patients guided to AKIT, F317L mutation observed patients are started to nilotinib and dasatinib treatment. *Conclusion.* To determine the optimal treatment type; resistance should be identified in early stages in CML patients, rational treatment type should be planned according to the determined mutation type.

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THE CORRELATION BETWEEN THE MEDIUM ERYTHROCITARY VOLUME AND THE BCR-ABL/ABL RATIO IN PATIENTS WITH CHRONIC MYELOID LEUKEMIA

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Background. Even if imatinib mesylate is not acting on leukemic stem cells, which are commonly found in the quiescent state, its administration permits in most cases of chronic myeloid leukemia to obtain the disease control, which means a significant decrease in leukemic cell mass, reflected at the monitoring by RT-PCR by lowering the BCR-ABL/ABL ratio to less than 0.1% and sometimes even to the absence of its detection. *Aim.* We aimed to study which parameters of the patients with chronic myeloid leukemia who are in their first months of treatment with imatinib mesylate correlate with the lowest BCR-ABL/ABL ratio they can obtain, parameters which in their turn could constitute prognostic factors for the response at the treatment with imatinib mesylate. *Methods.* We conducted a retrospective study which included all patients with chronic granulocytic leukemia who are in the records of the Hematology Department of the Emergency County Clinical Hospital from Sibiu who were treated with imatinib mesylate, at whom we have analyzed the following parameters: gender, age at diagnosis, the disease duration, the duration of treatment with imatinib mesylate, the hemoleucogram and the erythrocytes medium volume after 2-3 months and the lowest BCR-ABL/ABL ratio obtained by each patient, thereafter, during the treatment. We have compared each of the parameters mentioned with the BCR-ABL/ABL ratio, using the Pearson test. *Results.* The study included 21 patients with the mean age of 49.35±16.18 years. The women/men ratio was 8/13. The lowest average BCR-ABL/ABL ratio obtained over time was 0.60±1.27%. We have found a statistically significant inverse correlation between this ratio and the erythrocytes medium volume (93.41±7.63 fl) (r=-0.598). In other words, the higher the erythrocytes medium volume is in the first 2-3 months of administration of imatinib mesylate, there are more chances for the BCR-ABL/ABL ratio to reach a low value, in dynamics. There was also found a direct correlation between the mentioned ratio

and the platelet count (212904.8±171212.6/mm^3) (r=0.346) and between that ratio and age (r=0.334). So, as the platelet count is higher in the first 2-3 months of administration of imatinib mesylate, the chances are higher for the BCR-ABL/ABL ratio to remains high, in dynamics. Patients with advanced ages have more often a high BCR-ABL/ABL ratio. The BCR-ABL/ABL ratio did not correlate with gender, disease duration, the duration of treatment with imatinib mesylate or with the parameters of the hemoleucogram, except platelets. *Conclusions.* The increased erythrocytes medium volume in the first months of imatinib mesylate administration correlates with a future low BCR-ABL/ABL ratio, suggesting that it could be a positive prognostic factor for obtaining, in dynamics, the molecular response. Conversely, a large number of platelets in the first months of administration of imatinib mesylate are correlated with a future high BCR-ABL/ABL ratio, suggesting that it could be a negative prognostic factor for obtaining subsequent molecular response.

1354

THE EFFICACY OF IMATINIB MESYLATE TREATMENT PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA PH' POSITIVE (CML) REFRACTORY TO PREVIOUSLY INTERFERON THERAPY (CML) - THE FOLLOW UP OF OWN STUDY REPORT

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Imatinib Mesylate is the selective inhibitor of BCR / ABL tyrosine kinase of first generation presented at the most patients with CML. *The aim of study.* analysis of efficacy and safety Gleevec therapy in 112 CML Ph' positive patients resistant to previously given therapy i.e. Interferon and others (Hydrea, Mercaptopurine, ARA-C, Vincristine, Epirubicine, relapse after BMT). *Methods.* 112 patients were eligible for the 96 months study: - 108 patients in chronic phase-CP of CML, - 04 patients in accelerated phase-AP of CML. The patients received Gleevec in a daily oral dose of 400 mg in CP and 600 mg in AP of CML . Doses were modified (400-600mg daily) in a case of side effects or an incomplete achieved hematologic response. *Results.* Complete hematologic responces were reported for 97% evaluated CML treated patients after 2-3 months therapy and complete cytogenetic responses for 41.1% (46/112) CP-CML patients after 8-14 months has been lasting 34 months. There were maintained the primary hematologic resistance in 2.6% (3/112) patients, and the cytogenetic ones in 20.5% (23/112). The acquired hematologic resistance were in 5.3% (6/112) pts., and cytogenetic ones in 60.7% (68/112) patients. During study partial cytogenetic response (PCR) was observed in 15.2% (17/112) after 4.5 -37.0 mo (median 8.1 mo), which has been lasting 14.0 - 71.0 mo (median 22.3 mo+). Minimal cytogenetic response lasting median 4.0 mo was received in 43.8 % pts.(49/112) after 6.0 mo Imatinib therapy. Gleevec is well tolerated. Side effects (1-4o WHO) were observed at 23.2 % (26/112) patients : leucopenia, neutropenia, rash, hypoplasia medullae ossium, thrombocytopenia, bone and abdomen pain, increased AST/ALT level, allergic reactions, fever, hiperhidrosis. Survival time since Imatinib mesylate therapy is 27.5 -116.0 months for CML-CP patients.

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THE EFFICACY OF NILOTINIB TREATMENT PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA PH' POSITIVE (CML) REFRACTORY TO PREVIOUSLY IMATINIB MESYLATE THERAPY - THE OWN STUDY REPORT

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Nilotinib is the selective inhibitor of BCR / ABL tyrosine kinase of second generation which can be used at the treatment CML patients refractory to previously Imatinib mesylate with CML. *The aim of study.* analysis of efficacy and safety Nilotinib therapy in 30 CML Ph' positive patients resistant to previously given therapy i.e. Gleevec or Dasaninib. *Methods.* 30 patients were eligible for the 36 months study: - 28 patients in chronic phase-CP of CML, - 02 patients in accelerated phase-AP of CML. The patients received Nilotinib in a daily oral dose of 800 mg in CP and AP of CML . Doses were modified (800-400mg daily) in a case of side effects. *Results.* Complete hematologic responces were reported for 90% (27/30) evaluated CML treated patients after 1-2 months therapy and complete cytogenetic responses for 16.7% (5/30) CP-CML patients after 6-12 months has been lasting 24.8+months. There were maintained the primary hematologic resistance in 6.7% (2/30) patients, and the cytogenetic ones in 3.3% (1/30). The acquired hematologic resistance were in 0.1% (3/30) pts., and cytogenetic ones in 3.3% (1/30) patients. Major molecular response (CMR+PMR) were maintained at 3.3% (1/30) patients. During study partial cytogenetic response (PCR) was observed in 16.7% (5/30) after 6.0 mo (median 8.1 mo), which has been lasting 6 - 27 mo (median 16.5 mo+). Minimal cytogenetic response lasting median 4.0 mo was received in 33.3 % pts.(10/30) after 6.0 mo Nilotinib therapy. Nilotinib is good tolerated. Observed side effects At 20% patients (6/30): neutropenia, thrombocytopenia, increased AST/ALT level, allergic reactions. Survival time since Nilotinib therapy is 2.0 -35.0 months for CML-CP patients.

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IS THERE ASSOCIATION BETWEEN GASTROINTESTINAL STROMAL TUMORS AND CHRONIC MYELOID LEUKEMIA? EXTRAORDINARY EFFICACY OF IMATINIB IN BOTH DISEASES

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Background. Gastrointestinal stromal tumors (GISTs) are KIT-positive mesenchymal tumors of the gastrointestinal tract that are driven by activated KIT-signalling or platelet-derived growth factor receptor-alpha (PDGFR) signaling. These tumors most commonly occur in the stomach and small intestine and encompass a clinical spectrum from benign to malignant. In Targeting constitutively activated tyrosine kinases, such as BCR-ABL, in chronic myeloid leukaemia (CML) and c-KIT in GIST has substantially changed the clinical management of both diseases. The introduction of imatinib, a tyrosine kinase inhibitor mainly targeting BCR-ABL, c-KIT and PDGFR, has profoundly improved the prognosis of both entities. Imatinib induces cytogenetic and molecular responses in CML and also has substantial activity in GIST patients. Thus, in view of the low rates of severe toxicities and the extraordinary efficacy of the drug in both diseases, imatinib represents an oral drug with a high benefit-risk ratio for the treatment of CML and GIST. We report the unusual case of a patient who was diagnosed simultaneously two tumors whose common treatment is Imatinib. We conducted a review of the literature and found that there is an association, between GIST and CML (not statistically significant). *Matherial and Methods.* We reported the following case: a 63-year-old man patient that developed simultaneously CML and small intestine GIST. Patient with a history of arterial hypertension and hypothyroidism. In an analytical performed as preoperative presented: WBC 23960 (mielemia and basophilia) 12.4 g/dL hemoglobin 495000/mm^3 platelets. The bone marrow biopsy was compatible with chronic myeloproliferative syndrome, type CML. The patient was operated of a retroperitoneal mass of 18×15×9 cm, attached the inferior vena cava, right kidney and duodenum. Retroperitoneal GIST diagnosis is high grade malignancy. After diagnosed as high risk GIST, the patient was treated with imatinib at a dose of 400 mg/day, and the tumor was controlled. Follow up during 7 months, patient is alive, good performance status, and chronic phase CML with mayor molecular response. *Review of literature.* Miettinen M. *et al*. (Cancer. 2008;112: 645-9) examined long-term follow-up data of 1892 GIST patients. Nine patients (2 with gastric GISTs and 7 with GISTs of the small intestine) developed myeloid leukemia. There were 6 patients with AML, including 1 case of promyelocytic and 1 case of myelomonocytic leukemia, and 3 patients with CML. The leukemias developed 1.7 to 21 years after the GIST (median interval, 6 years). None of the GIST patients had received radiotherapy or chemotherapy prior to the leukemia diagnosis. Eight of 9 patients died of leukemia, and none died of GIST. In GIST patients, the risk of AML was found to be significantly higher for women and overall. There was a slightly increased risk for CML, but this was not statistically significant. *Conclusions.* Additional epidemiologic, clinical, and pathogenetic studies are needed to understand the apparent nonrandom association between GIST and myeloid leukemia. In this moment, therapy of both entities has successfully changed.

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COMPLETE MOLECULAR REMISSION WITH PLATELETS NUMBER NORMALIZATION AFTER DASATINIB THERAPY IN PATIENT WITH CML COMPLICATED BY THROMBOCYTOPENIA AND RESISTANT TO IMATINIB DUE TO AN E459K MUTATION

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Imatinib mesylate, an inhibitor of BCR-ABL tyrosine kinase at a dai-

ly dose 400 mg is a gold standard of first line treatment in patients with chronic myeloid leukemia (CML). This therapy is generally well tolerated, but in 20-50% myelosupression including thrombocytopenia is observed. These events are associated with temporary dose reduction or drug discontinuation, what may be cause of disease progression and promotion of leucaemic clone with BCR-ABL kinase domain mutation. The hope on effective therapy in such cases gives us the second generation of tyrosine kinase inhibitors. We present a patient (pt) with CML complicated by thrombocytopenia after imatinib therapy and disease progression because of E459K mutation successfully treated with dasatinib. *Case report.* A 61 year-old man with Ph-positive CML was treated with imatinib at 400 mg daily after a 5-months period of hydroxycarbamide therapy. After 3 weeks of such therapy thrombocytopenia in grade 3 acc. WHO (42×10^9/L) was developed. The dose of imatinib was decreased to 300 mg. The lower dose (200 mg) was also applied temporary due to thrombocytopenia intensification (35×10^9/L). The cytogenetic analysis of bone marrow after 6 and 12 months of imatinib therapy revealed 40% and 10% of Ph-positive metaphases, respectively. Such level of cytogenetic response (Ph 10-15%) was maintained during 3 years, when progression with increase of Ph-positive metaphases to 45% was detected. Direct sequencing of BCR-ABL kinase domain displied an E459K point mutation. Therefore the decision to change of therapy on dasatinib was made. The initially dose of dasatinib was low (40 mg daily) and was increased gradually to 100 mg. That was possible because platelet number was increasing systematically to complete normalization after 6 months of such therapy. In that time cytogenetic analysis revealed complete response. After 12 months of dasatinib therapy complete molecular response in RT-PCR analysis was achieved. *Conclusions.* The BCR-ABL kinase domain mutations are the most frequent causes of resistance to first-line imatinib therapy. Sensitivity of mutated leucaemic clone on dasatinib or nilotinib was established on basis of *in vitro* analysis. There are only a few reports concerning the clinical outcome of pts with mutations treated by second generation of TKI. Our case presents high sensitivity of leucaemic BCR-ABL-positive clone with E459K mutation on dasatinib, which additionally overcome thrombocytopenia after imatinib therapy.

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IMATINIB MESYLATE IN NEWLY DIAGNOSED CHRONIC MYELOID LEUKEMIA (CML) PATIENTS

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Background. Imatinib Mesylate (IM) is a powerful and selective p210 Bcr-Abl tyrosine kinase inhibitor that has been demonstrated to be effective for the treatment of CML. *Aims.* The study was performed to investigate the safety, efficacy, tolerance and compliance of IM in newly diagnosed CML patients. *Methods.* From June 2004-June 2008 we collected 62 untreated Ph^+ CML patients. They received Imatinib at diagnosis at a dose of 400 mg orally per day. 35 were male and 27 female with a median age of 45 (range 23-70 years) Sokal risk low 32,4%, intermediate 47.5% and high 20.1%. *Results.* Complete hematological remission was achieved in 95% patients. The estimated rate of complete cytogenetic remission (CCR) was 75,7% after 12 months. The most commonly reported adverse events after IM were edema (including peripheral and periorbital edema) (55 %); muscle cramps (29.7%); diarheea (25.5%); fatigue (31.8%); moreover in 4,2% patients with grade 3-4 events were observed consisting of pancitopenia and/or elevated liver enzymes. Four patients died: 1 patient of cardiac infarction, 1 patient of metastatic colon cancer and 2 patients of blastic crisis. In summary, at 84 months, 96% patients are alive and 83% of them are still receiving IM. 13 patients discontinued Imatinib: 7 patients for adverse events grade 3-4 and 6 patients for progressive disease. *Conclusions.* These data confirm the safety and efficacy of IM in newly diagnosed patients.

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THIRD LINE THERAPY WITH NILOTINIB INDUCED FIRST MAJOR MOLECULAR RESPONSE (MMR) IN A PATIENT WITH CHRONIC MYELOID LEUKAEMIA AND F317L MUTATION

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Introduction. This report describes the case of a patient affected by chronic myeloid leukaemia (CML) with F317L mutation of the Bcr-Abl domain, responding to nilotinib after acquiring resistance to imatinib and dasatinib. *Case report.* In 1996, a forty-three years old woman was diagnosed with CML and treated with alfa-Interferon (alfa-IFN), achieving complete haematological response (CHR). Three years later, patient was switched to hydroxiurea due to thyroid toxicity. For logistic reasons, therapy with imatinib (400 mg/die) was initiated only in 2003, obtaining complete cytogenetic response (CCyR) and suboptimal molecular response in twelve months. CCyR and CHR were then lost three years later. Doubling imatinib dose to 800mg/die gave no positive *Results.* Mutational analysis performed in September 2007 showed F317L point mutation of the Bcr-Abl kinase domain. In October 2007 dasatinib was started and in April 2008 CCyR was reached with suboptimal molecular response. In March 2009 Bcr-Abl transcript progressively increased, and in August 2009 cytogenetic analysis showed loss of CCyR. In the same month, therapy with Nilotinib 800mg/die was started, and in October 2009 the patient obtained MMR. *Discussion.* The reported case shows successful third-line treatment with nilotinib in a CML patient proven to bear the F317L mutation after developing resistance to imatinib. In particular, patient had never obtained MMR before receiving nilotinib. Bcr-Abl kinase-domain point mutations, acquired during first line therapy, are a common cause of resistance to tyrosine kinase inhbitors (TKIs). While several Bcr-Abl mutations have been identified, involvement of codon 317 has been reported in the literature following treatment with imatinib and dasatinib. A study by Jabbour *et al*. described incidence, prognosis and response to therapy in F317L mutated patients. Prognosis seems to be independent from the type of mutation, and rather dictated by CML phase and response to salvage therapy. (Jabbour *et al.*, Blood 2008,210:4839-4842). Moreover, *in vitro* studies show high sensitivity of F317L mutated cells to nilotinib (O'Hare T, *et al.* Blood 2007;110: 2242-2249). *Conclusion.* Nilotinib has significant clinical activity in patients who failed therapy with other TKIs; mutational analysis of Bcr-Abl kinase-domain can help choosing effective TKIs and overcoming resistance.

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CHRONIC EOSINOPHILIC LEUKEMIA

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Background. Chronic Eosinophilic Leukaemia (CEL), a rare entity, is a myeloproliferative variant of Hypereosinophilic Syndrome defined by FIP1L1 gene rearrangements. Increased number of eosinophils are thought to mediate an inflammatory response by releasing contents of its secondary granules and hence responsible for the pathogenesis. Hepatosplenomegaly, eosinophilia with systemic disturbances, anaemia or thrombocytopenia and especially cardiac involvement are major components of the disease. In the past, prognosis was grave and most of the patients died especially from cardiac involvement but detection of gene rearrangements and their susceptibility to tyrosine kinase inhibitors has made a revolutionary change in prognosis and life expectancy of these patients. *Case report.* A thirty-two year old man who had been in good state of health previously was referred to our clinic with leucocytosis and high eosinophil count. He had fatigue, blurred vision, non-productive cough and sore throat for ten days; his physical examination revealed splenomegaly 10 cm below costal margin and eye examination revealed segmentation and dilatation of capillaries around the optic disc. His hemogram revealed the following; Hg:9.2 gr/dL, WBC:50000/μL Plt:98000/μL and eosinophil 21100/μL and on peripheral blood smear eosinophil 62 %, neutrophil 23 %, lymphocyte 10 %, metamyelocytes 3 % and myelocytes 2 %. In bone marrow aspiration, more than 50 % myeloid cells were eosinophils and blast ratio is below 3%. T (9; 22) with fluoresan *in situ* hybridisation method was 100% negative. Tests for BCR-ABL and FIP1L1-PDGFRA rearrangement with real time PCR were performed. Parasite tests for eosinophilia were negative. CT of the thorax and abdomen revealed splenomegaly and hepatomegaly. On follow-up, hypotensive attacks with dynamic electrocardiographic (ECG) changes were observed and methylprednisolon 1 mg/kg/day therapy started when pericardial effusion was seen on USG. Despite steroid therapy, his eosinophil count did not change as expected but hypotensive attacks and ECG changes were not seen again and pericardial effusion resolved. Erythrocyte and thrombocyte transfusions were needed on follow-up. BCR-ABL rearrangement was found negative however; FIPL1-PDGFRA rearrangement for RNA was found to be positive so Imatinib 100 mg/day began and steroid therapy quickly stopped. His medical condition quickly

improved, eosinophilia disappeared, vision improved and he was free of transfusions. He was discharged with good state of health and on his last outpatient visit, his Hg: 12.3 gr/dL, WBC: 5600/μL Plt: 148000/μL and eosinophil 0.4%. *Discussion and conclusion.* CEL, once disease with a poor prognosis, is now a manageable disease with a good quality of life with tyrosine kinase inhibitors. FIP1L1-PDGFRA fusion gene was found to be 50 fold more sensitive to Imatinib, a tyrosine kinase inhibitor, than BCR-ABL so lower doses of the drug (100 mg/day) had been found to be mostly successful in achieving hematologic and molecular response quickly. Our patient depended on transfusions and he had vision loss attributed to eosinophilia but after the start of Imatinib therapy, his medical and hematologic condition quickly improved. Here, we report our case presented with non specific symptomatology and rapid disease progression but good response to therapy in the end.

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CALCIUM AND PHOSPHATE METABOLISM WAS ALTERED IN CML PATIENTS RECEIVING DASATINIB

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Background. Dasatinib is an inhibitor of BCR-ABL and SRC-family kinases for patients with Imatinib-resistant or -intolerant chronic myelogenous leukaemia (CML). Recent studies have demonstrated that patients taking Imatinib had indicators of altered bone and mineral metabolism. We evaluated if Dasatinib therapy may also produce these adverse events. *Methods.* Transversal analysis of CML patients treated with Dasatinib from April 2007 to April 2008: 4 patients, with a median age of 57 years, in treatment with a median daily Dasatinib dose of 112 mg. All four patients were previously treated with Imatinib for a median period of 37 months. Serum levels of calcium, phosphate (nephelometry) and parathyroid hormone (quimioluminiscence) were determined. Eleven patients (median age: 58 years) with myeloproliferative sindromes Philadelphia chromosome negative served as internal controls. Qualitative variables were analyzed by Chi-squared or Fisher's Exact and quantitative variables by Mann-Whitney test. *Results.* Among the 4 patients receiving Dasatinib, one of them developed hypophosphatemia (P=0,011), another one hypocalcemia (P=0,01) and 3 of them hyperparatiroidism (P=0,004). In the same way, median values also differ significantly from those in the control group: phosphate (2,7 *vs.*3,5 mg/dL, P= 0,01), calcium (8,8 *vs.* 9,5 mg/dL, P= 0,01) and paratohormone (109 *vs.* 47 pg/mL, P= 0,01). One of the four patient didn´t show any anomalies. *Conclusions.* 1. Dasatinib therapy develops calcium and phosphate metabolism alterations. 2. Futher studies are necessary to investigate the effect of Dasatinib on bone and mineral metabolism.

No Conflict of Interest. Informed consent was obtained.

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CLINICAL OUTCOME AND PRESENTING FEATURES OF CD20 POSITIVE REED-STERNBERG CELLS CLASSICAL HODGKIN'S LYMPHOMA

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Background. Hodgkin and Reed-Sternberg cells (HRS) cells are the neoplastic population in classical Hodgkin's lymphoma (cHL). Recent immunological and molecular studies have shown that HRS cells originate from mature germinal centre B cells. Although cHL is genotypically considered a B-cell lymphoma, the classical B-cell marker CD20 is expressed with reported frequencies of 5-58%. The prognostic significance of CD20 expression in HRS cells of cHL is still controversial. *Aims and Methods.* To further assess the presenting features and the prognostic significance of CD20 expression in cHL, we performed a retrospective single institution study of 87 cases with a mean clinical follow-up of 12 years. The mean ages were 40.4 years (range, 14-83 years) for the men and 34.3 years (range, 16-77 years) for the women. Sixty-five (74.7%) of the 87 patients (pts) were younger than 45 years of age. The histology was nodular sclerosis in 75, mixed cellularity in 10, lymphocyte rich cHL in 1, and lymphocyte-depleted in 1. Stages III+IV were present in 57 pts (65.5%), bulky disease in 34 pts (39.1%), extranodal disease in 16 (18.4%), 51 pts (58.6%) had an IPS score 0-2 (low-risk) and 36 (41.4%) had a score of ³3 (intermediate-high-risk). Combined radiochemotherapy was administered in 57 pts (65.5%) and chemotherapy alone in 30 (34.5%); 24 pts (27.6%) received the MOPP/ABVD, 16 (18.4%) the BEACOPP, and 47 (54%) the ABVD chemotherapy regimen. *Results.* HRC expressed CD20 in 27 pts (31% of cases). The negative rate of CD20 was significantly higher in patients with B-symptoms (54% *vs.* 32.2%, P=0.046) and with bulky disease (49.4% *vs.* 22.9%, P=0.035). There was no statistically significant difference in CD20 expression between the groups with other different clinical parameters. No significant difference in terms of response rate (82% *vs.* 85%) was found between CD20 positive and negative cHL. The 5-year progression free survival (PFS) rates were 76.2% in CD20-positive patients and 82.2% in CD20-negative patients (P=n.s.; Figure 1). The 5-year overall survival (OS) rates were 91.4% in CD20-positive patients and 92.5% in CD20-negative patients (P=n.s.). *Conclusions.* CD20 is expressed by HRS cells in 31% of patients with cHL; it is higher in patients without bulky disease and B symptoms. However, according to our results, the expression of CD20 is not an independent prognostic factor for PFS and OS of naive cHL patients.

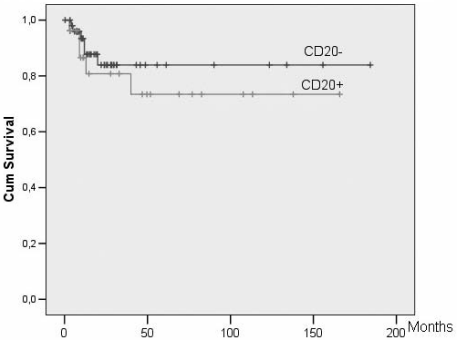

Figure 1. PFS in pts with cHL according to CD20 expression in HRS.

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THE TEI-INDEX EVALUATION: A MORE EFFECTIVE ECHOCARDIOGRAPHY MONITORING IN PATIENTS WITH HODGKIN'S AND NON-HODGKIN'S LYMPHOMA

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Background. Anthracyclines are widely employed in lymphoma's chemotherapy and has been shown to induce heart failure. To monitor this toxic damage, echocardiographic parameters of left ventricular (LV) systolic function are usually used. *Aims.* of this study was to evaluate in lymphoma's patients the reliability of echocardiographic data in comparison with a LV systo-diastolic parameter function: the Tei-Index. *Methods.* Twenty-nine pts (10 males, 19 females, mean age: 49 yrs) suffering from Hodgkin's and non Hodgkin's lymphoma respectively treated with ABVD or R-CHOP at standard dose. We performed a two-dimensional doppler echocardiography before the first and the fourth chemotherapy cycle, calculating in apical 4 chamber view: LV end-diastolic volume (EDV), end-systolic volume (ESV), ejection fraction (EF) and stroke volume (SV). During mitral and aortic pulse doppler flow, we evaluated the Tei-Index, according to the next formula: Tei-Index = (isovolumetric relaxation time + isometric contraction time)/ejection time. *Results.* We divided the patients in two groups, according to the presence (Tei+, 9 pts) or the absence (Tei-, 20 pts) of a Tei-Index increase (cut off: 0.1). EF did not change in either group (Tei: 63.35% *vs.* 62.34% P=.0809; Tei+: 63.02% *vs.* 64.78% P=.2638). In Tei- group EDV, ESV and SV increased from the first to the fourth cycle (EDV: 63.23 mL *vs.* 70.88 mL P=.0172; ESV: 23.55 ml *vs.* 26.92 ml P=.0012; SV: +10.78% P=.0809). In Tei+ group EDV and ESV did not change (EDV: 73.16 ml *vs.* 68.59 mL P=.2426; ESV: 27.35 ml *vs.* 24.34 mL P=.2177) and we did not observed a significantly reduction of SV (-7.47%; P=.3666). *Conclusion.* According to our data, it seems that SV increase in Tei- pts by increasing the LV volumes. This phenomenon could be explained by early "positive"LV remodelling. On the contrary, Tei+ pts seem not able to counterbalance at the same way and SV reduces, in spite of an unchanged EF. We believe that Tei-Index could be a useful parameter to diagnose the compensative ability of the heart muscle to toxic damage by anthracycline, earlier than EF. Nevertheless, we still have to understand if the remodelling phenomenon observed in Tei- pts could lead to a better prognosis during the follow-up.

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EXPRESSION OF CANCER-TESTIS ANTIGENS IN HODGKIN'S LYMPHOMAS USING TISSUE MICROARRAY

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Background. Cancer-testis (CT) antigens are expressed in a variety of malignant tumors and in normal adult tissues solely in testicular germ cells. Owing to this tumor-associated expression pattern, these antigens are potential targets for immunotherapy. *Aims.* To analyze the expression of CT antigens in Hodgkin's lymphomas (HL) using tissue microarray (TMA), and to correlate their expression with histologic subtypes, clinical parameters such as gender, disease stage, EBV status and treatment response. *Methods.* Formalin-fixed paraffin-embedded tissues of 38 HL cases were obtained from the archives of the Department of Pathology, Federal University of São Paulo, Brazil. All cases were classical HL, of the following subtypes: 25 (65.8%) nodular sclerosis, 9 (23.7%) mixed cellularity, 2 (5.2%) lymphocyte-rich and 2 (5.2%) not specified. 20 patients were male, 18 female; EBV was detected in 17 patients. A TMA block was constructed including two cores of each tumor sample, and the following monoclonal antibodies (mAbs) were used for immunohistochemical stainings: "MAGE-A cocktail"consisting of mAbs MA454, M3H67, 57B and 6C1, to allow screening for MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6 MAGE-A10 and MAGE-A12; mAb CT7-33 (to CT7/MAGE-C1), mAb CT10#5 (to CT10/MAGE-C2), mAb E978 (to NY-ESO-1) and mAb #26 (to GAGE-family antigens). Staining was carried out using the avidin-biotin peroxidase complex, and two independent observers scored all slides. *Results.* Eight (21.1%) of 38 HL cases expressed at least one CT antigen, which were 6/25 nodular sclerosis and 2/9 mixed cellularity. Among the 8 positive cases, 6/20 were female and 2/18 male. According to Ann Arbor stage, 6/21 advanced stage (III or IV) and 2/17 early stage (I or II) patients were CT antigen positive. Of 17 EBV-positive cases, 4 were showed CT antigen expression, and 4/21 EBV-negative cases were also CT antigen positive. CT antigen expression were observed in 6/30 patients who achieved complete response and in 2/8 who had refractory disease. Among the tested CT antigens, MAGE-A and CT7 were the most frequently expressed in HL, being present in 7/38 (18.4%) and 5/38 (13.2%) respectively. *Conclusions.* Using a panel of antibodies to detect the expression of MAGE-A, CT7, CT10, NY-ESO-1 and GAGE antigens, we found that 21.1% of HL cases express at least one CT antigen. Interestingly, in the present series CT antigen expression appears to be higher in advanced stage disease than in early stage disease. MAGE-A family and CT7 were the most frequently expressed CT antigens in HL. Our data suggest that CT antigens could serve as diagnostic markers and/or potential therapeutic targets in a proportion of classical Hodgkin's lymphomas.

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ASSOCIATION OF EPSTEIN-BARR VIRUS AND HODGKIN'S LYMPHOMA: PREVALENCE AND LONG TERM SURVIVAL

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The association of Epstein-Barr virus (EBV) with Hodgkin's lymphoma (HL) has been investigated over the last few years. The impact of EBV on clinical outcome is still controversial, however. In this study, we investigated the effect of EBV status on clinical outcome of Algerian HL patients (Pts). This study included sixty-eight Pts with HL (33 females, 35 males), all clinically staged during 1990-1992. Median age was 25 years (6"70). Histological subtypes included lymphocytic predominance (LP) 3; nodular sclerosis (NS) 33; mixed cellularity (MC) 30 and lymphocytic depletion (LD) 2. According clinical staging, 47% where localized forms (IA, IIB). EBV markers examined included expression of latent membrane protein (LMP) in Reed-Sternberg and Hodgkin cells (RSC) by immunochemistry. The LMP was expressed RSC in 26 Pts. LMP was significantly associated with MC subtype (1 LP, 8 SN and 17 MC). The viral expression was more frequent in the localized forms of HL. The LMP was expressed in respectively 50% and 28% of IA-IIB and IIIA-IVB stages. The ten years overall survival was respectively 81% and 62% for HL LMP positive and HL LMP negative (P=0.003). EBV infection may be involved in the pathogenesis of HL in our Algerian study cohort and it does significantly affect therapeutic results and overall survival of HL Pts.

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BEACOPP-14 VS BEACOPP-ESC IN PATIENTS WITH HODGKIN'S LYMPHOMA FROM POOR-PROGNOSIS GROUP: PRELIMINARY RESULTS OF PROSPECTIVE RANDOMIZED MULTYCENTER STUDY

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Background. At the moment, it is unclear which of therapy approach is optimal for patients with high risk Hodgkin lymphoma (HL). For this reason many clinical trials undergo comparing different chemotherapy and radiotherapy regimens. Promising results demonstrates of BEACOPP-14 regimen. The prospective randomized multicenter study comparing two regimens in patients with high risk HL was started in Ukraine. The aim of study: compare the treatment efficacy and toxicity BEACOPP-14 and BEACOPP -esc. regimens in patients with high risk HL. *Methods.* from September 2008 until now 65 patients from 18 to 65 years old (median 29 year), 26 male and 39 female with stage IIB with more than one unfavorable factor and stage III-IV were included in the study. All patients were randomized to receive BEACOPP-14 (31 patient, totally received 185 cycles, 6,02±1,08 cycles per patient) and BEACOPP-esc (31 patient, totally received 206 cycles, 5,96±1,08 cycles per patient). The treatment efficacy in both groups was evaluated after 4, 6 and 8 cycles by Cheson criterias (1999). For patients who achieved complete response after 4 cycles, maximum 6 cycles was done. Toxicity rate was evaluated with NCI-CTC V.3.0. Chemotherapy in patients with initial sites >5 cm, residual lymph nodes >2 cm and/or PET-positive sites was followed by radiotherapy (30-36 Gy). *Results.* completion of overall response after 4, 6 and 8 cycles of chemotherapy is presented on the diagram. The treatment of patients from high risk HL with BEACOPP-14 and BEACOPP-esc is highly efficient. The rate of complete response after 4 cycles of BEACOPP-14 was higher (66,6%) than after the 4 cycles of BEACOPP-esc (52%) (P>0,05); after 6 cycles (81,25% and 64,7%, respectively) (P>0,05). After 8 cycles 100 % overall response rate was reached in both groups), complete response rate was equal (88,8 % and 87,5%, respectively) (P>0,05). Maximal observation period is 12 months. Positive response is keept in all patients, cases of disease progression were not detected. The main toxicity was haematological toxicity of different stages, that was detected in 69,3% of chemotherapy cycles totally (67,55% during the treatment with BEACOPP-esc in comparison with 72,8% during the treatment with BEACOPP-14, P>0,05). Anemia was detected in 69,29% cases (BEACOPP-esc - 57,74%, and BEACOPP-14 - 82,1%) (P<0,05). Neutropenia was detected in 67,51% cases (BEACOPP-esc - 64,4 %, BEACOPP-14 - 70,7%) (P<0,05). Febrile neutropenia was detected in 10,1 % cases (BEACOPP-esc - 10,1 %, BEACOPP-14 - 8,6%). Thrombocytopenia was detected in 31,45 % cases (BEACOPP-esc - 33,49%,BEACOPP-14 - 29,18%) (P<0,05). The most frequent non-hematologic toxicity were nausea and vomiting, that were detected in 44,85 % of patients (BEACOPP-esc - 54,5%, BEACOPP-14 - 35,2%). Mucositis was detected in 18,8% cases during the treatment with BEACOPP-esc and in 19,3% during the treatment with BEACOPP-14. *Conclusion.* Both comparative regimens BEACOPP-esc and BEACOPP-14, are effective (100% overall response after 6-8 cycles) and showed equal toxicity rate. However, the results are preliminary and should be confirmed in larger number of patients and with a longer follow-up.

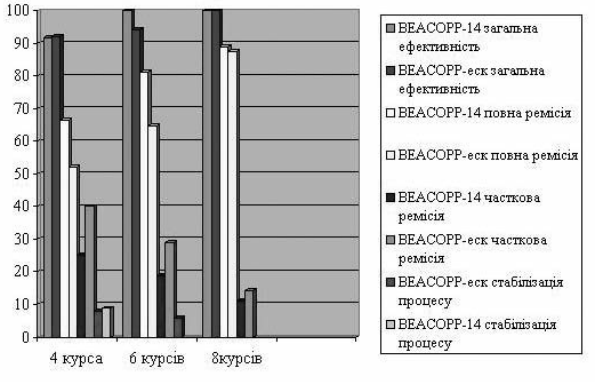

Figure. Completion of overall response.

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HIGH-DOSE THERAPY IN CHEMOREFRACTORY HODGKIN'S LYMPHOMA PATIENTS

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It has been shown that patients (pts) with chemosensitive relapsed and refractory Hodgkin's lymphoma (HD) were more likely to remain progression-free after high-dose therapy (HDT) and autologous stem cell transplantation (auto-SCT). We report here retrospective analysis evaluating the outcomes of 45 pts who didn't response to salvage therapy or progressed before HDT. Indication to HDT is controversial in this chemorefractory group but it corresponds with our transplant program. *Patient and Methods.* In total 22 male (48.9%) and 23 (51.1%) female with progressive HD were treated with HDT and auto-SCT from February 2001 to October 2009. The median patient age was 28.9 years (range 13-54). The primary-refractory before salvage were 26 pts (57.8%), others - relapsed. The average number cycles of previous therapy were 9 (range 3 - 30), by radiotherapy treated 35 pts (77.8%). Pts who received badly standardized previous therapy (inadequate estimation of responses, extended treatment intervals, reduced doses and so on) - 18 (40%). Were performed 51 auto-SCT and one allo-SCT. Median numbers of CD 34^+ cells reinfused were 6.6×10^6/kg (range 1.97-18.8×10^6/kg). Salvage regimens were mainly platinum-based, they were received by 42 pts (93.3%). More than one line pretransplant salvage therapy received 15 pts (33.3%). Preparative HDT mainly was CEAM (lomustin 400 mg/m^2 instead carmustin) - 35 pts (68.6%). *Results.* After a median follow-up of 19.3 months (range 2.2 - 99.9) the overall survival (OS) is 65,4% (CI 95% 50,6%-80,2%), and progression free survival (PFS) is 43,5% (CI 95% 28,3%-58,7%). The most frequent causes of death were disease progression - 76.2% of cases. TRM in estimated group was 6.67%, while in chemosensitive group was 2.7%. Our assumption was that the worst results will in group of initially inadequately treated patients, but we have not found difference in OS and PFS. Significantly better (P=.00521) survival (OS and PFS) was observed in part of patients which was reinfused more than 10.0×10^6/kg CD 34^+ cells in comparison with less 5.0×10^6/kg. *Conclusions.* This prognostic unfavorable category of patient also must be included in transplant programs. Number of infused stem cells can influence an outcome.

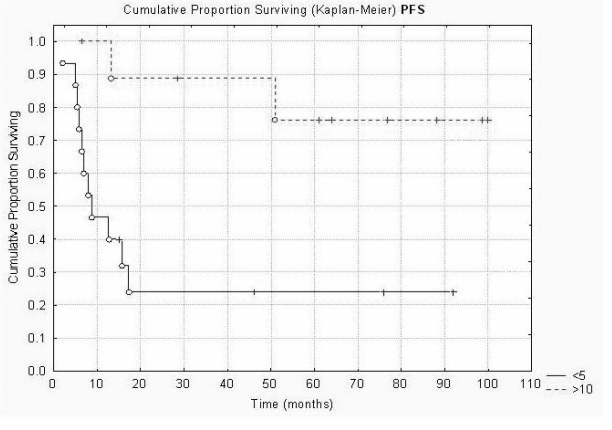

Figure.

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CUTANEOUS HODGKIN'S DISEASE: A RARE ENTITY

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Introduction. Hodgkin's disease involving the skin is an unusual occurrence and usually occurs late in the course of Hodgkin's lymphoma. This rare condition is thought to have decreased in incidence in recent decades, likely owing to improved treatment of patients with Hodgkin's disease, who are receiving improved chemotherapy and radiation therapy, and the advent of peripheral blood stem cell transplantation. The most common clinical presentation is of single or multiple dermal or subcutaneous nodules. Direct extension from an underlying nodal focus, hematogenous dissemination, and most often, retrograde lymphatic spread, distal to involved lymph nodes, are the mechanisms usually implicated. We report the case of a patient with Hodgkin's disease who presented with skin involvement at diagnosis. *Case report.* we present a case of an 85 years old woman who developed Hodgkin's lymphoma with cutaneous involvement. She accused malaise, fatigue and loss of weight. On physical examination the unilateral supraclavicular lymph node was found (diameter 2.0 cm) and ulcerated lesion of the neck of 5 cm diameter. The remaining of physical finding was normal. Laboratory data revealed: RBC $4,8\times10^6$/μL, Hgb 13.5 g/dL, WBC $6,2\times10^3$/μL, PLT 242×10^3/μL; renal functions, Beta 2 microglobulin, Ves, copper, fibrinogen and ferritin were normal. LDH was high: 679 UI/L (v.n.300-600). Total body scan was negative. The biopsy of lymph node was performed and the histology reveled Hodgkin's lymphoma, mixed cellularity subtype. The PET total body after node biopsy showed a single area with excavation at the canter at the level of subcutaneous tissue in the left occipital (SUV max 5.9). The skin biopsy pointed a dermal localization of Hodgkin's lymphoma, $CD30^+$ and $CD15^+$. Histology of bone marrow was normal. The patient was considered in clinical stage II AE. Chemotherapy with VEPEMB protocol (Vinblastina,procarbazina,prednisone,etoposide,mitoxantrone, bleomicina) was started; the patient received 3 full cycles obtained a complete remission, proved by clinical, biochemistry, and PET/TC imaging techniques. After chemotherapy RT/IF was performed. Discussion: Primary cutaneous HD is very unusual and usually represents a rare late manifestation of dissemination of the disease heralding a grave prognosis. Specific skin lesions were described in 3.4-7.6% from all cases of HD and have been categorized as papules, nodules, plaques or infiltration, ulcerative lesions, a combination of these, and erythroderma and often are accompanied by pruritus. The skin of the chest seems to be most frequently involved. Many cases of coutaneous HD can be excluded from other cutaneous granuloma by the immunohistochemistry criteria for this disease. More recently, cutaneous HD has been described in patient with human immunodeficiency virus (HIV) infection, advanced clinical stage, and aggressive clinical course, suggesting that alteration in immune system function is important for disease appearance and spread. EBV positively has been previously described in cutaneous HD in only one case. *Conclusion.* Cutaneous involvement usually represents, or accompanies, Stage IV Hodgkin's disease, and often portends an ominous prognosis. Nevertheless, it also might follow a relatively benign course, and more and more intensive systemic chemotherapy sometimes is effective in such cases.

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HODGKIN'S LYMPHOMA IN HONG KONG CHINESE

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Hodgkin's lymphoma constitutes about 30% of all cases of lymphoma in Caucasian population.[1] However, a lower incidence of 6.1% was found in Hong Kong adult patients.[2] An analysis of patient characteristics, clinical course and treatment outcome of Hong Kong Chinese patients in a local hospital was done. *Methods.* It was a retrospective study of Hodgkin's lymphoma patients in a local hospital from 1991 to 2009. Data were collected from old notes and hospital computer file. Patients were staged according to Ann Arbor Staging system. The response rate and overall survival were assessed and the overall survival was calculated by Kaplan-Meier method. *Results.* Sixty-six Chinese patients with Hodgkin's lymphoma were reviewed. There were 37 males and 29 females and the median age was 37 (range 19-81 years). Most of the patients had the histology subtypes of nodular sclerosis (52%), followed by mixed cellularity (39%), nodular lymphocyte predominant Hodgkin's lymphoma (7.6%) and lymphocyte rich (1.5%). There was no case of lymphocyte depleted histology. There were 26% of patients with stage I disease, 41% with stage II, 24% with stage III and 9% with stage IV disease. There was a bimodal age distribution with peak incidence at 21-30 and 61-70 years of age. A variety of treatments were given to these patients (chemotherapy alone, radiotherapy alone and combined treatment). Patients also received various chemotherapy regimes including ABVD, ChlVPP, stanford V, escalated BEACOPP. ABVD was the most commonly used regimen (68%). Radio-

therapy alone was also given to some patients with early stage disease. The complete remission rate was 80% and the advanced stage had a lower CR rate (33% for stage IV, 56% for stage III, 93% for stage II and 100% for stage I disease). The 5-year overall survival for the patients was 85%. Age and advanced stage (III/IV) are independent variable significantly affecting the overall survival. Sex of patient is not a significant factor for the survival. *Discussion.* The relatively low incidence of Hodgkin's lymphoma has been a consistent finding. Orientals may have a relatively high incidence of T-cell lymphoma, especially NK-T cell lymphoma. The distribution of histologic subtypes was quite similar to the pattern found in Caucasians with nodular sclerosis being the commonest histology. The incidence was higher in male patients. There was also an early peak in young adults and another peak at advanced age. The treatment regimen was changing. Combined modality treatment incorporating chemotherapy especially ABVD with or without radiation was used for most patients. The complete remission rates and overall survival in this study were satisfactory. The major cause of death of the patients was refractory lymphoma. *Conclusion.* Hodgkin's lymphoma has a lower incidence in Hong Kong Chinese than in Caucasians. Nodular sclerosis and mixed cellularity are the commonest histologic subtypes. Age and staging were independent variables affecting the overall survival.

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DISEASE CHARACTERISTICS AND TREATMENT APPROACH AS PREDICTIVE FACTORS IN HODGKIN'S DISEASE

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Labeling Hodgkin's disease (HD) as one of the curable neoplastic diseases, motivates hematologists to define the basis on which this contemporary medical achievement is accomplished. Our study is a contributing attempt in this direction. At our Clinic we have been managing more than 500 HD patients over a 25-year period. We have studied the prognostic impact that disease onset manifestations, mostly included in the IPI scoring system, but alongside with and across different treatment approaches, and the impact that both have on the disease course and its outcome. Of a total of 473 HD evaluable patients, 119 were with clinical stages 1-2A, and were analyzed regardless of the disease mass (bulk). Evaluable advanced stage patients, with clinical stages 2B-4, also irrespective of tumor mass size, were 348 of them. Multivariate statistical analyses show that of the seven IPI parameters, only the scoring system backbone still retains its prognostic significance: Hb and Alb levels. Patient's gender, and a modified range for age and clinical stage of the disease, also remain important factors. Selective statistics reveal that this importance is valid mostly due to factor's impact on advanced stages of HD, whereas the prognosis of early stage HD patients does not seem to be affected by these parameters. The values for the chi-square tests are 45.9023 and 24.5866 for 3 degrees of freedom (gender, Hb, Alb) for the whole population and the advanced disease subset respectively, and the p-values are highly significant (0.00000 and 0.00002), when the analysis is performed on standard IPI values. Modified values achieve even higher significance and incorporate more parameters. Following our assessment that onset manifestations do not have statistical influence on the outcome of early stage disease patients, we extended the analysis to the post-diagnostic period. Analyzing different types of treatment engaged, as well as similar approaches, it is clearly evident that these patients benefit from treatment modalities containing the gold standard and the increasingly competitive newer chemotherapy regimen: ABVD and BEACOPP. Interestingly enough, and possibly due to the low number of entries, combined modality treatment did not show significant advantage over chemotherapy alone in these early stage patients. On the other hand, advanced stage disease course could not be significantly altered by employing even more aggressive treatment approaches. Rough grouping of the originally and contemporarily treated subsets, show an evident difference of 25-40% in overall survival ($P<0.001$). Both observations clearly imply that rapid diagnosis, diminishing the possibility of disease advancement, and utilization of contemporary and aggressive treatment options in the first instance, speculating that the appreciated effect is mostly attributable to the potential of doxorubicin and very possibly etoposide, are the milestones on which the successful story of HD management has been created and confirmed.

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RETROSPECTIVE STUDY OF HODGKIN LYMPHOMA IN A 10-YEAR PERIOD AT A COMMUNITY HOSPITAL

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Background. Hodgkin's Lymphoma (HL) affects mostly young people. Old people and patients in advanced stage have a worse outcome. *Aims.* To analyze the clinical data, outcome and treatment toxicity of HL patients during a 10-year period at a 450-bed hospital. *Patients and Methods.* Retrospective analysis of the clinical features, therapy and outcome of patients with HL treated between January 1999 to October 2009. *Results.* Seventy one patients were identified. They were 45 (63%) male and 26 (37%) women; median age was 42 years (range 17-89) and seventy five % were younger than 45 years. Histology was: 41 (58%) nodular sclerosis, 14 (20%) unspecified HL, 7 (10%) nodular lymphocyte predominant HL, 6 (8%) mixed-cellularity and 3 (4%) lymphocyte-rich. Characteristics of the disease at diagnosis were as follows: early stage 39 (55%), advanced stage 32 (45%). Seven patients (7%) had Bulky disease, 33 (46%) had B symptoms and performance status measured by ECOG scale was 0-1 in 63 (89%). Thirty eight (97%) of the 39 patients in early stage received treatment: 27 (69%) combined modality (ABVD plus Radiotherapy), 10 (26%) AVBD alone and 1 (2.5%) Radiotherapy alone. After therapy, responses were as follows: 35 (92%) complete response, 1 (3%) partial response and progression in 2 (5%) patients. Among patients in advanced stage (32), 30 received chemotherapy (28 AVBD, 1 BEACOPP and 1 DHAP); four patients received also radiotherapy. In this group 25 patients (83%) achieved a complete response, 2 (7%) partial response and 3 were not evaluables (1 was lost of follow-up and 1 died respectively before evaluation and 1 patient was already in course of treatment at the moment of analysis). In terms of toxicity, grade 3-4 neutropenia was observed in 19 (28%) of patients and grade 3-4 infections in 6 (9%): 4 patients with febrile neutropenia, 1 fungal pneumonia and 1 patient suffered an acute hepatitis B reactivation. In 6 (9%) patients pulmonary-toxicity was observed (grade 1-2 in 2 and grade 3-4-5 in 4 patients). Three patients developed grade 3-4 cardiac toxicity. One patient presented a second malignancy (breast cancer). Nine patients relapsed/progressed and required new line of treatment. Among those, 5 were in advanced stage and 4 in early stages (all of them had any unfavourable risk factor according the EORTC). No relapses were observed in the early favourable-stage group). Eleven (15%) patients died and the causes of death were as follows: 6 (8.5%) disease progression, 3 (4%) toxicity (1 pneumonia and 2 pulmonary toxicity; all of them were older than 70 years) and 2 patients died for others reasons (1 patient aged 92 years for pneumonia after remaining 5 years in complete response, and 1 presented a sudden death). With a median follow-up of 41.5 months (range 1-123) overall survival in the Kaplan Meier's curves was 100% in early favourable-stage, 80% in early unfavourable-stage and 78% in advanced stage. *Summary.* In our cohort of HL patients the responses were according to the previously described in the literature. Old people presented the more severe toxicity.

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THE LEVEL OF ADENINE NUCLEOTIDES AT HODGKIN LYMPHOMA BEFORE AND AFTER CHEMOTHERAPY

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Considering the significance of adenine nucleotides (AN) and adenosine in proliferation and differentiation of immune cells we have studied AN metabolism in patients with Hodgkin lymphoma before and after chemotherapy. The aim of the work was to study AN pool and the state of the processes of their decay (activity of 5'-nucleotidase) in peripheral blood lymphocytes at Hodgkin lymphoma before and after the therapy. The AN content was studied by the method of thin-layer chromatography, and the activity of ecto-5'-nucleotidase - by spectrofotometric method. An acute (almost 2 fold) decrease in ecto-5'-nucleotidese activity was found in lymphocytes, accompanied by a significant (1,3 fold) rise of AMP level on the background of some ($P<0.01$) decrease in ADP quantity and stable level of ATP. The qualitative and

quantitative interchanges of the components of adenylic system led to a marked decrease in the ATP/AMP and increase in ATP/ADP ratios (P<0,01), while the energetic charge (EC) of the cells did not undergo any deviations. Thus, at Hodgkin's lymphoma despite the stability of EC, marked alteration of ATP/AMP and ATP/ADP ratios is observed probably due to the disturbance of ecto-5'-nucleotidase activity. After chemotherapy a definite normalization of the studied indices occurs. The question of informativity of the indices of the exchange of ecto-5'-nucleotidase activity and metabolites of adenylic system at Hodgkin lymphoma is discussed, as of additional prognostic criteria and for assessment of the conducted treatment efficiency.

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PLATINUM-BASED PROTOCOLS IN THE TREATMENT OF RELAPSED OR REFRACTORY HODGKIN'S DISEASE

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Background. The platinum-based protocols (ESHAP or EDHAP) have been widely used in relapsed or refractory cases of non-Hodgkin lymphoma (NHL) or as a conditioning regimen prior to transplantation as well. In contrast to these data, their use in Hodgkin disease is scanty. *Aims.*- We present our experience in 18 patients with relapsed or refractory Hodgkin disease treated with these protocols, sixteen of them prior to transplantation. *Methods.* Eighteen patients (8 males, 10 females), mean age 32 years (17-58) are presented. Histologic subtypes included 3 lymphocyte predominance, 12 nodular sclerosis and 3 mixed cellularity. Eight cases were relapsed and 10 resistant to previous therapies. Fifteen patients were treated with ESHAP cycles and only one with EDHAP. No modifications on the conventional doses or schedule of these protocols were made. In another two cases with lymphocyte predominance histology Rituximab (375 mg/m^2, day 1) was added to the conventional protocol (ESHAP and DHAP, one each). *Results.* A total of 57 cycles were administered, mean number of cycles was 3,2 (2-5). Seventeen patients received complete doses and in only one case corticosteroids were reduced 50% due to previous hypertension. No delayed administration of cycles was observed. Toxicity included: Anemia in 15 cases (83,3%), mean grade 1,9 (1-4); neutropenia in 11 (61,1%), mean grade 3,7 (2-4); thrombocytopenia in 12 (66,7%), mean grade 2,8 (1-4); mild increases of creatinine levels in 3 (16,7%); hypomagnesemia in 6 (33,3%); febrile syndrome in 1 (5,5%); emetic syndrome in 5 (27,7%). Response was observed in 14 cases (77,7%) (5 CR and 9 PR). Sixteen patients received an hematopoietic progenitors transplantation post-platinum treatment: Fifteen autologous transplantation, and the other a non-myeloablative allogenic one from his sister. In those cases of autologous procedures enough number of CD34$^+$ cells (data from 14 patients: mean 8,3×10^6/Kg; 38-18) could be obtained from peripheral blood (13 cases) or bone marrow (1 case) without significative problems, mean number of aphereses 1,48 (1-2). Two patients (autograft) have relapsed after transplantation (one patient is now being transplanted). *Conclusions.* In our experience, platinum-based protocols could be a safe, generally well tolerated and worthwhile option for the treatment of patients with refractory or relapsed Hodgkin disease. Toxicity is mainly related to cytopenias and mild renal failure. These protocols do not seem to affect the number of CD34$^+$ cells collected in cases of autologous transplantation.

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THE 'BATTLE' FOR THE GOLD STANDARD IN HODGKIN'S DISEASE: EVIDENCE FOR SUPERIORITY OF ABVD IN THE PRE-BEACOPP ERA

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Different treatment approaches were available and utilized over time for patients with HD. Even today, different centers and schools use different treatment modalities as standard. Efforts are directed towards tailoring the optimal treatment method for different risk stages. Nevertheless, in the segment of chemotherapy, most researchers still accept the ABVD regimen as the present and valid 'gold' standard for treating all stages and types of HD, in combination or without adjuvant radiotherapy. In a single-center study, on a population of more than 500 HD patients, treated at our Clinic over a longer than 25-year period, we attempted to define points where ABVD has shown superiority over earlier chemotherapy option(s), the sustainability of prognostic models for HD patients when analyzed separately according to treatment protocol, as well as the benefit it implies, evaluated by patient longevity and quality of life. Univariant statistical analyses were performed on a number of patient characteristics, disease manifestations and treatment modalities and their outcomes, and compared in patients grouped according to treatment protocol, meaning also according to the period when they were observed and/or treated at our Clinic. Special attention was placed on the subgroup of HD patients in whom the ABVD chemotherapy protocol was the principal one. Multivariate analyses were performed in order to define whether known predictive factors, certainly the ones incorporated in the IPI, and others, sustain prognostic significance within the 'newer' subpopulation of HD patients, treated initially with the present gold chemotherapy standard. The most obvious advantage of ABVD treated patients was observed in the field of life expectancy. Overall survival was above 85% at 10 years, reaching its plateau shortly after year 2, as opposed to a near 55% for MOPP-based treated patients. EFS followed this line of *Conclusions.* Chance for relapse was also markedly low. Patients treated with the hybrid MOPP/ABV(D) were not evaluable for the analyses, since when this treatment option first became available, it was widely used for poor risk HD patients, thus diminishing their chances for a privileged outcome. In the segment of univariate statistical analyses, most of the parameters maintained their prognostic significance, although with slightly higher p-values. This applies to the IPI parameters also, both with the original settings, as well as under our dichotomization criteria (slightly altered points derived from population and disease characteristics). Nevertheless, in multivariate analyses, ABVD treated patients manifest no outcome affection by the known predictive parameters, except for a couple: one patient characteristic - age, and one disease manifestation - symptom presence. Therefore, HD patients treated with ABVD chemotherapy as their underlying regimen, still remain with significantly greater outcome expectations and very high reliability for achieving definite disease control. In that process not many patient characteristics, nor disease manifestations can influence their favorable prognosis, which is why this chemotherapy protocol is applicable to patients with different age and gender, with disease of different type or extent, with comparably similar favorable effect. The evidently lower incidence of undesired late effects is also a fact deserving respect and a confidence vote.

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EFFECTIVENESS OF DECITABINE IN CHRONIC MYELOMONOCYTIC LEUKEMIA PATIENTS. INTERNATIONAL COOPERATIVE MULTI-CENTER STUDY

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Background. Epigenetic therapy with hypomethylating agents has recently been approved for the treatment of myelodysplastic syndromes (MDS) and chronic myelomonocytic leukemia (CMML) in South Korea and Argentina. CMML is a heterogeneous disorder sharing features of MDS and Chronic Myeloproliferative disorders. It has recently been reported that decitabine (DACOGEN, Janssen Cilag Farmaceutica S.A. and Eisai Inc.) is effective in the management of CMML. *Aims.* To describe the clinical and hematological improvement with decitabine among patients with CMML on a 'real world program'. *Methods.* This field investigation was based on data collection from patients with CMML who received decitabine at different centers of South Korea and Argentina between July 2007 and December 2009. Inform consent has been signed according to the regulations of each institution. A report prepared ad hoc was completed. We took into account WHO classification, as well as performance status by ECOG, co-morbidities, previous treatments and IWG 2006 criteria. Efficacy was evaluated with at least 2 cycles. Inclusion criteria were 18 years of age and confirmed diagnosis of CMML type 1 or type 2. Exclusion criteria were diagnosis of acute myeloid leukemia (AML) or other progressive malignant disease. Patients with prior therapy were not excluded. All patients received decitabine 20 mg/m^2 IV over 1 hour once daily for 5 consecutive days repeating every 4 weeks. We evaluated the overall improvement rate (complete response + marrow complete response + partial

response + hematologic improvement) and rate of stable disease or better. *Results.* The study population included 33 CMML patients (M/F: 24/9 ; median age 67, range 23-82). CMML Type-1 63.6 (21) %, Type-2 36.4(12) %. Karyotype was normal 75.8% (n=25), isolated -7/7q- 6.1% (n=2), +8 6.1% (n=2), del3q/der3 3.0% (n=1), tY/1 3.0% (n=1), complex 3.0% (n=1) and no metaphases 3.0% (n=1). The median interval from diagnosis to treatment was 3 months (R 0 - 35); The median number of cycles received was 5 (R 1-12) with a median follow-up of 14 months. According to IWG 2006 criteria, the overall response rate (ORR) was 43%, including CR in 8 (26.7%), marrow CR (mCR) in 1 (3.3%), PR in 0 (0 %), stable disease (SD) in 5 (16.7%), HI in 4 (13.3%), and progression in 9 (30%). Clinical and Hematological response are summarized in Table. Most of the patients remained alive during the first year of follow-up. 2 patientS received allogeneic stem cell transplant without additional toxicity. *Conclusions.* Decitabine is an effective and generally well tolerated treatment in CMML. This treatment strategy allows relatively young patients to be transplanted in a better clinical condition and can be the best option for older patients.

Table.

Treatment response	
Complete response (CR)	8 (26.7%)
Partial response (PR)	0 (0%)
Marrow complete response (mCR)	1 (3.3%)
Hematologic improvement (HI)	4 (13.3%)
Stable disease (SD)	5 (16.7%)
Overall improvement rate (CR+PR+mCR+HI)	13 (43%)
Rate of stable disease or better	18 (60%)
AML development	9 (30%)
Failure	12 (40%)

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PTPN11 MUTATIONS WERE RARE IN PATIENTS WITH MYELODYSPLASTIC SYNDROMES AND CHRONIC MYELOMONOCYTIC LEUKEMIA

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Background. PTPN11 gene, encoding SHP-2, is the disease gene of Noonan syndrome. Mutations of PTPN11 have been described in children with juvenile myelomonocytic leukemia, myelodysplastic syndromes (MDS) and acute leukemia. It is not known that the role of PTPN11 in adult MDS and chronic myelomonocytic leukemia (CMML) and the role in disease progression to acute myeloid leukemia (AML). *Aims.* The aims of this study are (1) to determine the prevalence of PTPN11, N-Ras, K-Ras mutations in adult MDS and CMML; (2) to correlate these mutations with time to progression to AML and overall survival (OS). *Methods.* Bone marrow samples from patients with MDS and CMML at diagnosis and at AML transformation were examined for N-Ras, K-Ras and PTPN11 mutations. Analysis of N-Ras and K-Ras mutations were performed by genomic DNA or cDNA PCR followed by direct sequencing. Analysis of PTPN11 mutations was performed by cDNA PCR followed by direct sequencing. *Results.* One hundred and twenty-one patients with MDS and 92 patients with CMML at diagnosis were available for analysis. Eighty-one patients with MDS and 38 patients with CMML also had samples at AML phase available for analysis. Among patients with MDS, three had N-Ras mutations at diagnosis and 8 had N-Ras mutations at AML phase. None had K-Ras mutations at diagnosis but 3 acquired the mutations at AML phase. None had PTPN11 mutations at diagnosis and at AML phase. The time to AML transformation and OS were not statistically different between patients with and without N-Ras, K-Ras, or PTPN11 mutations. Among patients with CMML, sixteen had N-Ras mutations at diagnosis and 9 at AML phase (3 of them did not have mutations at diagnosis). Three patients harbored K-Ras mutations at diagnosis. One patient gained K-Ras mutation at AML phase. Four patients (4.3%) had PTPN11 mutations at diagnosis and 2 of them retained the same mutations at AML phase. The time to AML transformation and overall survival (OS) were not statistically different between patients with and without N-Ras, K-Ras, or PTPN11 mutations. However, CMML patients with N-Ras mutations had the trend of inferior OS than those without the mutations (P=0.068). We found that N-Ras, K-Ras, and PTPN11 mutations were mutually exclusive in patients with MDS or CMML. *Conclusions.* Our results showed that N-Ras mutation played a role in the AML transformation and no patients had PTPN11 mutations at diagnosis and at AML phase in patients with MDS. PTPN11 mutations were rare in patients with CMML. There was no statistical difference in term of time to AML transformation and OS according to the status of N-Ras, K-Ras, and PTPN11 mutations in patients with MDS and CMML.

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COMPARING THE PROGNOSTIC VALUE OF DIFFERENT TYPES OF PHENOTYPIC ABNORMALITIES OF HEMOPOIETIC PRECURSORS IN MYELODYSPLASTIC SYNDROMES

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Background. several phenotypic alterations of bone marrow (BM) hemopoietic precursors have been described in myelodysplastic syndromes (MDS). Increase and abnormal phenotypes of $CD34^+$ cells have been associated with a worse outcome of the patients. *Aim.* to compare the influence on survival of several kinds of phenotypic abnormalities with that of peripheral cytopenias and IPSS. *Methods.* BM of newly diagnosed patients with MDS was examined by multiparametric flow cytometry examining the myelomonocytic lineage and the hemopoietic progenitors in four-color combinations. The influence on overall survival of age, PB counts, IPSS, total number of phenotypic abnormalities, abnormalities of granulocytic and monocytic precursors as well as BM percentage of blasts in cytology and subsets of $CD34^+$ cells were analyzed using the Cox model. *Results.* We analyzed 55 cases: 6 RA, 2 RARS, 30 RCMD and 17 RAEB. IPSS: 22 cases were low risk, 20 intermediate-1, 7 of intermediate-2 and 6 were high risk. Median follow-up time: 23 months (1-43). In the univariate analysis, the following variables were associated with a shorter survival: aggressive WHO type (P=0.001), a higher IPSS score (P=0.004), the degree of anemia (P=0.007), a higher BM blast percentage in cytology (P<0.0005), but not age, PB neutrophils and platelets. Among the flow cytometric variables, the total number of phenotypic abnormalities (P=0.004) and that of $CD34^+$ cells (P=0.002), as well as the percentage of $CD34^+$ cells (P<0.0005), $CD34^+/CD13^+$ (P=0.0006) and $CD34^+/CD13^-$ cells (P=0.0002), besides $CD34^+/CD19^+$ cells among all nucleated cells (P=0.05). The number of granulocytic aberrancies and the number of dendritic cell precursors had no influence on survival. In the multivariate Cox regression comparing IPSS with PB counts and phenotypic abnormalities, only IPSS and the number of abnormalities of $CD34^+$ cells were independent risk factors. In a model comparing only the percentage of blasts in cytology and each single abnormality of $CD34^+$ cells stratified by WHO type, only the percentage of blasts in cytology and the increase of $CD34^+/CD13^-$ cells were independent risk factors for a shorter survival. *Conclusions.* specific phenotypic changes in MDS, rather than their total number are important risk factors in MDS. Among them, the most important were the changes in $CD34^+$ cells which reflect the degree of genetic disarrangement of hemopoietic precursors producing a maturation block of the abnormal clone.

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FAK EXPRESSION IN MYELODYSPLASTIC SYNDROME AND ACUTE LEUKEMIA

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Background. Myelodysplastic syndrome (MDS) encompasses a heterogeneous group of clonal hematopoietic stem cell disorders characterized by ineffective hematopoiesis, refractory cytopenia and a tendency to progress towards acute myeloid leukemia (AML). The occurrence of acute leukemia results from a combination of mutations and changes in protein functions that confer the ability of proliferation, defects in cell differentiation and apoptosis. The identification of cellular signaling pathways responsible for the increase in survival and reduction in cellular differentiation of myelodysplastic cells is of great importance, because these signaling pathways may represent therapeutic targets in an attempt to prevent progression to AML. Focal Adhesion Kinase (FAK) is a key mediator of signaling induced by inte-

grins and plays an instrumental role in cell survival and proliferation. Increased FAK expression in acute leukemia was associated with resistance to Danorrubicina therapy and poor prognosis, however, no study of the expression or function of FAK in MDS has been performed. *Aims.* The aim of the present study was to investigate the gene and protein expression of FAK in MDS, AML and ALL patients and to correlate the level of expression with low risk *vs.* high risk MDS according to FAB. *Methods.* We studied 09 healthy donors, 27 patients with MDS (18 low risk [RA/RARS] and 09 high risk [RAEB/RAEBt] according to FAB classification), 14 patients with AML (07 M2, 01 M3, 03 M4, 02 M5, 01 M6, according to FAB classification) and 10 patients with ALL (5 T ALL and 5 Pre B ALL). Samples were obtained from total cell of bone marrow. Gene expression was evaluated by real time RT-PCR in total cells. Western blot of mononuclear cell of bone marrow (patients) or 34^+ cell of peripheral blood (healthy donors) was performed for protein expression *Results.* Real time RT-PCR demonstrated a significant decrease in FAK expression of AML cells compared with normal haematopoietic cells (0.10 [1.22-0.005] *vs.* 0.29 [1.00-0.08], P=0.04) or with low risk MDS cells (0.10 [1.22-0.005] *vs.* 0.36 [1.42-0.01], P=0.01); no differences in FAK expression was detected between low and high risk MDS cells (0.36 [1.42-0.01] *vs.* 0.16 [2.39-0.01], P=0.19) and when comparing high risk MDS cells or ALL cells with normal haematopoietic cells (0.16 [2.39-0.01], P=0.34 or 0.10 [1.16-0.01], P=0.13 *vs.* 0.29 [1.00-0.08], respectively). Western blot analysis revealed absence of FAK expression in AML samples, but a positive expression was detected in $CD34^+$ cells as well as in ALL patient samples, confirming real-time *Results. Conclusion.* FAK is down-regulated in AML cells. The inclusion of a higher number of high risk MDS would be necessary in order to point out the involvement of FAK in disease progression of MDS towards AML. This work was supported by FAPESP and CNPq.

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COEXISTENCE OF MYELODYSPLASTIC SYNDROMES AND PLASMA CELLS DISORDERS. A CYTOGENETIC REVIEW

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Background. Myelodysplastic syndrome (MDS) is a hematological malignancy characterized by dysplasia and ineffective hematopoiesis. The lineage affected in this pathology is the myeloid one. Plasma cells disorders (PCD) affect B-cells derivates, producer of immunoglobulines, the most common being monoclonal gammopathy of undetermined significance (MGUS) and; the most classic, the multiple myeloma (MM). The coexistence of both pathologies is a rare event, although, the appearance of a MDS following plasma cells pathology is more frequent. In this study, we describe the cytogenetic characteristics of a series of 15 preliminary cases with the coexistence of both MDS and a PCD at presentation. *Patients and methods.* Reviewing cases with the diagnosis of PCD and MDS from centers belonging to the Spanish Hematological Cytogenetics Working Group (GCECGH) and to the MDS International Cytogenetics Working Group, we recruited 42 cases. Among them, 27 presented a MDS during the evolution of the PCD, and 15 presented a coexistence of PCD and MDS at diagnosis time. We have collected cytogenetic information: conventional cytogenetics in short term culture and FISH 5q result (in case it was performed). In some cases in which the FISH was performed on plasma cells, have been also reported. Results. The following table shows the diagnoses, conventional cytogenetic and FISH (Fluorescence *in situ* Hybridization) information from our series of 15 patients. *Conclusions.* The following preliminary conclusions can be highlighted: 1- The short term culture showed in all cases karyotype corresponding to MDS, but one case with a complex karyotype (more close to the PCD). 2- MDS karyotype presented similar findings and incidence of chromosomal abnormalities than cases with *de novo* MDS, although in two cases trisomy 8 and deletion 20q were observed. 3- From eight patients in whom the FISH analysis on 5q was performed, half of them revealed aberrations of chromosome 5, not previously observed by conventional cytogenetics. However, trisomies are more typical of PCD, suggesting hyperdiploidy. This work is presented on behalf of the Grupo Cooperativo Español de Citogenética Hematológica (GCECGH), and the International Working Group on MDS Cytogenetics of the MDS Foundation. *Acknowledgements.* This work has been partially supported by grants from Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo, Spain: FI07/00107.

Table.

ID	MDS	PCD	Karyotype (short term culture)	FISH 5q
1	CMML*	WM	46,XY[20]	Negative
2	MDS	LPL	46,XY[20]	Negative
3	CMML*	MGUS	46,XX[20]	Not done
4	RARS	MM	46,XY[20]	Trisomy
5	RA	MM	46,XX[20]	Negative
6	RCMD	MM	46,XY[20]	Deletion
7	MDS	MGUS	46,XX[10]	Not done
8	MDS	MM	46,XY[10] *[del(13q),del(17p),+3]***	Not done
9	RAEB-t*	MM	46,XY[10] *[t(6;14),+9,+11]***	Not done
10	MDS	MM	Not done *[del(13q),t(14;20),+3,+4,+11,+16,+17,indicating hiperdiploidy]***	Not done
11	RA	MM	Not done	Trisomy
12	MDS	MM	46,XY,del(20)(q11)	Not done
13	MDS	MGUS	47,XX,+8,del(12)(p11)[7]/46,XX[13]	Negative
14	RAEB	MM	46,XY,del(20)(q12)[8]/47,idem,+8[1]/46,XY[1]	Not done
15	RAEB-1	MM	Complex karyotype with an hyperdiploid clone	Monosomy and trisomy

* MDS according to the FAB classification. ** FISH performed in plasma cells.

Abbreviations: PCD: plasma cell disorder; CMML: Chronic Myelomonocytic Leukemia; WM: Waldenström's macroglobulinemia; LPL: Lymphoplasmacytic Lymphoma; MGUS: Monoclonal Gammopathy of Undetermined Significance; RARS: Refractory Anemia with Ringed Sideroblasts; MM: Multiple Myeloma; RA: Refractory Anemia, RCMD: Refractory Cytopenia with Multilineage Dysplasia; RAEB-t: Refractory Anemia with Excess of Blasts in transformation; RAEB-1: RAEB type 1.

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FLOW CYTOMETRY COUNTING AND ANALYSIS OF MYELOID AND NUCLEATED ERYTHROID CELLS WITH IRREGULAR ANTIGENIC PROPERTIES IN MYELODYSPLASTIC SYNDROME

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Background. Myelodysplastic syndrome (MDS) is a group of clonal stem cell disorders hallmarked by refractory cytopenia and blood cell dysplasia. Flow cytometry (FC) is a useful method to study hematopoiesis and detect myelodysplasia. The analysis of myelopoesis is the most informative part of FC analysis in MDS, particularly when the results of morphology or cytogenetic testing are ambiguous. FC finding of dysplastic myeloid cells in bone marrow is currently documented by abnormal distribution of cells with myeloid markers in different fluorescent dot plots. Unsatisfactory level of standardization and difficulty in interpretation of results limit the utility of FC in diagnosis of MDS. The identification and enumeration of dysplastic cells by FC in suspected MDS may help to standardize and simplify the diagnostic work-up. This approach to FC analysis of patients with MDS has not been tested, and its clinical significance remains unknown. *Study aim.* The present study was performed to evaluate the diagnostic significance of FC count of dysplastic myeloid and erythroid cells in MDS. *Methods.* The patient's cohort enrolled in this study consisted of 47 cases of MDS and joined control group composed of 10 cases of idiopathic cytopenia of undetermined significance (ISUS), 56 cases of disorders distinct from MDS and ICUS but with potential impact on bone marrow hematopoiesis (pathological controls), and 15 patients with normal BM (normal controls). Bone marrow myelopoiesis was studied using multicolor staining and specially designed analysis templates. The cells failed to pass the gates collecting normal cells, were classified as myeloid or erythroid cells with undetermined (irregular) antigenic characteristics (MUC/NEUC). These cells were collected and counted using logical (Boolean) gate. The ratio between myeloid and lymphoid progenitors, $CD117^+$ cell count and B-cell maturation index were the additional FC parameters measured in patients. *Results.* Cell analysis revealed an elevated number of MUC in 84% patients with MDS, comparing to

30% patients in joined control group (P<0.0001, Fisher's exact test). The specificity of MUC count was 78%, the sensitivity was 83% with negative predictive value 91%. Increased number of NEUC was found in 69% patients with MDS and in 40% of controls (P<0.02). The combination of these two tests for diagnosing MDS showed the precision rate of 89% and negative predictive value of 94%. The finding of elevated MUC number together with abnormal values of any two additional FC parameters had a significantly increased specificity of the test. *Conclusion.* The study demonstrated that FC may be used for detection and count of myeloid and nucleated erythroid cells with abnormal antigenic characteristics in MDS. The enumeration of dysplastic cells may simplify FC criteria for the diagnosis of MDS and facilitate diagnostic procedure in patients suspected for having MDS.

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5-AZACITIDINE FIVE DAYS/MONTHLY SCHEDULE IN SYMPTOMATIC LOW-RISK (IPSS: 0-1) MYELODISPLASTIC (MDS) PATIENTS. CLINICAL AND BIOLOGICAL EFFECTS

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Background. Nucleoside 5-Azacitidine (5-Aza) in high risk MDS patients (pts) at a dose of 75mg/mq/day subcutaneously for 7 days, every 28 days, induces high hematologic response rates (hematologic improvement (HI) 50-60%, complete remission (CR) 10-30%) and prolongation of survival (at 2 years 50,8%). *Aim.* The role of 5-Aza in low-risk MDS patients is not well defined but its use in the earlier phases of disease could be more effective and useful to control the expansion of MDS clone and disease progression. In our phase II, prospective, multicentric trial a low-dose schedule of 5-Aza (75 mg/mq daily for 5 consecutive days every 28 days) was given to low-risk MDS pts in order to evaluate its efficacy and tolerability and to identify biological markers to predict the response. *Methods.* From September 2008 to February 2010, 34 patients were enrolled into the study. Fifteen patients had refractory anemia (RA), 5 patients refractory anemia with ringed sideroblasts (RARS), 7 patients refractory cytopenia with multilineage dysplasia (RCMD) and 7 patients refractory anemia with excess blasts-1 (RAEB-1). All patients failed previously EPO therapy and were in chronic red blood cell (RBC) supportive care with a median transfusions requirement of 4 units/monthly. The response treatment criteria was according to IWG 2006. *Results.* At present time 31 out of 34 pts are evaluable: 12/31 pts (39%) completed the treatment plan (8 courses), 7/31 pts (22%) performed the first 4 courses, 8/31 (26%) made 1 to 3 courses and 4/31 (13%) died during the treatment period. Out of 12 pts who completed the 8 courses of therapy 10 (83%) obtained an HI, 2/12 (17%) maintained a stable disease. Out of 10 pts who obtained HI, 4 pts (40%) achieved a CR. Generally the drug was very well tolerated. The most commonly reported hematologic toxicities were neutropenia (55%) and thrombocytopenia (19%) but they were transitory and usually no delay of treatment was necessary. 2/4 pts died early after the 1th cycle for septic shock and gastrointestinal hemorrage respectively whereas 2/4 pts died in a condition of stable disease after the 4th cycle for pneumonia and respiratory distress. Samples for biologic studies have been collected from the pts before starting the therapy and at the end of 4th and 8th course. Preliminary data on the lipid signalling pathways suggested a direct correlation between PI-PLC-β1 gene expression and 5-Aza responsiveness. *Conclusion.* Interim analysis of our study based on the small number of cases who completed the treatment program, shows that 83% of pts obtain an HI and 40% obtain a CR. 4 patients died during the treatment and even if the causes were reported as no related to the therapy it has been considered that caution has to be reserved in given 5-Aza in these pts who are elderly and frail. Preliminary data of PI-PLC-β1 gene expression suggest that this and probably other biological markers could help us to know a priori who are the patients who have more chances to respond. *Acknowledgments.* this work was supported by MIUR 2008.

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ISOCHROMOSOME 17q AND DELETION 17p AS MARKERS OF GOOD CLINICAL RESPONSE TO DECITABINE IN MYELODYSPLASTIC SYNDROMES

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Background. Isochromosome 17q or *<i17q)* plays a presumably important pathogenetic role both in leukemia development and myelodysplastic syndrome (MDS) progression. p53 gene aberrations can result from an *<i17q)* or *del(17p)* in MDS patients with poor risk prognosis. MDS rarely displays a p53 mutation. The deoxycytidine analog decitabine not only is thought to reflect its ability to reactivate methylation-silenced genes, but also is known to induce a ribonucleotide reductase (p53R2/RRM2B), a robust transcriptional target, that correlates with good clinical response (Petra A Link, Cancer Res, 2008;8, 9358). *Aims.* To report clinical response to decitabine in MDS patients displaying *<i17q)* or *del(17p)*. *Methods.* Patients with *<i17q)* or *del(17p)* were analyzed from our international database made up of 99 MDS patients treated with decitabine as from July 2007 through December 2009. These patients displayed *<i17q*) or *del(17p)* as an isolate aberration or combined with at least 2 chromosomal alterations. We ruled out complex karyotypes (more than 3 cytogenetic abnormalities), and correlated chromosome findings with clinical response to decitabine that was infused at a dose of 20 mg/m^2/day in an outpatient setting. This study took into account WHO classification, International Prognosis Score System (IPSS) and International Working Group (IWG) 2006. *Results.* Four out of the 99 patients displayed *<i17q)*, in two cases as an isolate finding (patient #1 and #2), in another case (patient #3) combined with *del(5q)* and *del(7q)*; and patient #4 presented *del(17p)* combined with *del(11q)*. Baseline patient characteristics and clinical response are described in the Table below. All patients presented primary MDS, required transfusions before decitabine therapy, and had not received previous chemotherapy. Patient #1 and #2 had received erythropoietin and patient #4 had been treated with Thalidomide. There were no comorbidities except for patient #1, who suffered from ulcerative colitis. According to IPSS, patient #2 and #4 belonged in the high risk group, patient #3 in INT-2, and patient #1 in INT-1. Patient #1 achieved cytogenetic remission and is waiting for an unrelated bone marrow transplant. *Summary.* The *<i17q)* or *del(17p)* correlated *in vivo* with good clinical response in MDS patients under decitabine as it was described by literature mentioned above. This data highlights and guarantees further studies.

Table.

Patient	Age/ Sex	MDS Subtype	Karyotype	Nº cycles	Clinical Response
# 1	52M	RCMD	46,XY,i(17)(q10)[11]	7	Complete Response
# 2	64F	RAEB 2	46,XX,i(17)(q10)[20]	5	Stable Disease
# 3	71M	RAEB 2	46,XY,i(17)(q10), del(5q),del(7q)[9]	3	Hematology Improvement
# 4	79F	RAEB 2	46XX,del(17p),del(11q)[8]	8	Complete Response

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RISK ASSESSMENT IN MYELODYSPLASTIC SYNDROMES (MDS)

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Background. Myelodysplastic Syndromes (MDS) are clonal hematopoi-

etic stem-cell disorders characterized by ineffective dysplastic hematopoiesis involving one or more cell lineages, by peripheral-blood cytopenias and a high risk of progression to acute myeloid leukemia (AML). The slowly evolving process of neoplastic transformation explains the clinical, morphological and prognostic heterogeneity which is not sufficiently addressed even in current classification systems. *Aims.* We investigated the role of WT1 gene expression and its association with the expression of the chemokine receptor CXCR4 on bone marrow $CD34^+$ cells of MDS patients. *Methods.* BM samples from 46 MDS patients (according to WHO classification: 21 RA, 7 RAEB I, 4 RAEB II, 9 RARS, 3 deletion of 5q, 2 MDS unclass) were tested for WT1 expression at diagnosis and every 6 months. WT1 gene expression was evaluated by methods of real-time quantitative PCR (RQ-PCR). Surface CXCR4 expression were measured flow cytometrically. *Results.* At diagnosis, 22BM samples (10 RA, 6 RAEB I, 4 RAEB II, 1 RARS, 1 MDS unclass) expressed WT1 transcript amounts greater than the ranges level. The degree of WT1 expression was highly correlated with the type of MDS, was much higher in RAEB I and II compared with RA, and other types, and increased during disease progression. Moreover, a significant correlation was found between WT1 expression levels, blast cell percentage and CXCR4 over-expression on blast cells (as defined by CXCR4 mean fluorescence intensity ratio thresholds of more than 5). The patients received only a supportive therapy if necessary. After 6 months, 9 patients (2 RA, 5 RAEB I, 2 RAEB II) converted to AML. All of these patients showed at diagnosis an high WT1 and CXCR4 expression and a further elevation of WT1 expression level after 6 months. *Conclusions.* WT1 expression has been previously reported to be increased also in myelodysplastic syndromes. In this study, the data obtained show that in most MDS, including a large percentage of RA and almost the total number of RAEB I and II, WT1 is expressed above the range observed in normal controls in BM and that its expression is directly correlated with the type of MDS. A strong association is present between the level of WT1 expression and the blast percentage and the CXCR4 over-expression. The identification of a molecular marker so able to establish the tendency of MDS to progression can be of great help in decision making for MDS patients. Our results justify further investigation into the role of CXCR4 in MDS and suggest that WT1 and CXCR4 should be incorporated into the risk assessment of MDS patients.

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'STANDARD' DOSES OF RH-EPO ARE HIGHTLY EFFECTIVE IN IMPROVING ANEMIA IN SELECTED 'LOW-RISK' MYELODYSPLASTIC PATIENTS

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Background. Since more than twenty years recombinant human erythropoietin (rhEPO) has been widely used to correct anemia in patients with myelodysplastic syndromes (MDS). While first clinical trials -usually conducted with relatively low doses of rhEPO (ranging from 12.000 to 30.000 U per week)- showed favourable responses to rhEPO in no more than 15-20% of such population as a whole, in the last decade a better selection of MDS patients - i.e. reserving treatment to patients with low/intermediate-risk MDS - allowed for a relevant increase of the response-rate, ranging around the 40% of treated individuals; more recently, several studies demonstrated a favourable clinical response in an even higher percentage of low-risk MDS patients (proximally 50 to 65 %) using high-doses (up to 80.000 U weekly) of rhEPO. These treatments, however, are costly and not easily affordable in the long time. *Aims.* In order to verify if an accurate selection of MDS patients could results in a satisfying response-rate with a less expensive schedule of rhEPO, we prospectively explored the effects of "standard"doses of rhEPO in a cohort of anemic "low-risk"MDS patients. *Methods.* From January 2005 to December 2009 a total of 55 anemic (Hb <10 g/dL) consecutive patients (29 males; 26 females; median age 78 years) with low/intermediate-1-risk MDS (IPSS 0 -1) were treated after informed consent with rhEPO-alpha 40.000 U per week subcutaneously for at least 3 months; at the end of this period hematologic response was assessed; only patients achieving an hematologic response were allowed to continue treatment indefinitely; safety and efficacy of the treatment was recorded along the entire duration of the study. *Results.* According to IWG 2006 criteria, after 3 months of treatment 36 out of 55 (65.5%) patients achieved a favourable response to rhEPO; while the percentage of responders did not significantly differed according to different IPSS scores (67,7% with IPSS score 0 *vs.* 62,5% with IPSS score 0,5-1) response to rhEPO was significantly correlated with lower WPSS scores (90%, 53% and 42% of responders for WPSS 0, 1 and 2-3 scores, respectively). Treatment was discontinued because of unmanageable side effects in only one patient. Among the 36 responders, after a median follow-up of 38 months, 28 (77%) still maintain a hematologic response and continue the treatment. Progression to high-risk MDS or acute leukemia was observed in 7 out of the 55 patients after a median of 26 months. *Conclusions.* In summary, our study indicate that "standard"doses of rhEPO are at least equally effective as higher doses to induce durable hematological improvement in anemic patients with low-risk/intermediate 1 MDS; in this clinical setting, this schedule allows for a consistent reduction of costs of the treatment without precluding the achievement of potential benefit from a challenge with rhEPO.

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HETEROGENOUS PROGNOSIS OF 5Q ABNORMALITIES IN MYELODYSPLASTIC SYNDROMS IN KOREA

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Background. The myelodysplastic syndrome (MDS) associated with isolated 5q- and restricted with marrow blasts less than 5% was called '5q- syndrome'. This unique subtype of low-risk MDS with favorable prognosis is known as good responder of lenalidomide recently and occurs predominantly but not exclusively in middle age to older women in Western countries. In contrast, the patients with other abnormalities in chromosome 5 showed quite different clinical features from those with '5q- syndrome'. *Aims.* The aim of this study was a retrospective evaluation of clinical characteristics and prognostic impact of various factors in Korean patients with abnormalities in chromosome 5 including '5q- syndrome'. *Methods.* Among 456 patients with MDS diagnosed at 16 hospitals in Korea between 1996 and 2006, 370 patients with available cytogenetic data entered the study. Univariate and multivariate analysis including various prognostic factors were performed. *Results.* On the basis of chromosomal analysis, 93 out of 370 patients (25.1%) showed abnormalities in chromosome 5. The '5q-syndorme' was documented only in 10 patients (2.7%). The deletion of 5q was interstitial, of variable size, but with a predominance for 5q13-33 deletions (34.8%). The female predominance (P=0.029) was documented similar to Western country. The anemia was more prominent (P=0.003) and macrocytic (P<0.001) in '5q- syndrome' than other MDS patients. The erythroid hypoplasia in marrow seemed to be prominent in '5q- syndrome' (P=0.149). None of '5q- syndrome' was transformed to leukemia and overall survival was significantly better than other patients. (P=0.036). Thirty nine patients (41.9%) had various abnormalities in chromosome other than 5q deletion such as translocation with other chromosome or 5 monosomy. They didn't share the clinical features with '5q- syndrome'. The patients with mosaic chromosomes with isolated 5q- and normal chromosome also showed different clinical outcomes with '5q- syndrome'. *Summary/Conclusions.* The incidence of '5q- syndrome' in Korea was lower than that reported in Western countries. The favorable clinical features of '5q- syndrome' were also documented in Korean patients. Patients with isolated 5q- and excess blast (>5%), other abnormalities than isolated 5q-, or mosaic chromosome with isolated 5q- and normal chromosome didn't share the clinical features such as lower rate of leukemic transformation and long survival.

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DIAGNOSTIC UTILITY OF FLOWCYTOMETRY IN MYELODYSPLASTIC SYNDROME: A FIRST STUDY FROM INDIA

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Background. Flow cytometry is now being increasingly evaluated to define its apparently promising role in the diagnosis of myelodysplastic syndrome (MDS). Expert recommendations emphasize the need for further validation and standardization, including flow cytometric (FCM) evaluation of secondary myelodysplasia. In this first study from India we have used 5 - color flow cytometry to evaluate patients with MDS. *Aim.* To evaluate the role of flow cytometric immunophenotyping in diagnosis of myelodysplastic syndrome. *Methods.* Thirty four cases with unexplained cytopenias and suspected MDS were prospectively studied. Five - color analysis using Coulter FC500 was done on bone marrow (BM) samples without knowing the morphological diagnosis. These subjects were compared with normal controls (no leukemia or MDS) using primary gating of blasts, myeloid cells and monocytes according to CD45 expression and side scatter. Normal fluorescence intensity and maturation pattern was collected from 31 control BM: 3 hypersplenism, 17 idiopathic thrombocytopenic purpura, 4 lymphoma staging marrow, 6 post-chemotherapy regenerating marrows and 1 peripheral blood sample from donor of peripheral blood stem cell transplantation. A large panel using antibodies reported in the literature was employed including CD34, CD45, HLA-DR, CD38, CD117, CD33, CD13, CD64, CD14, CD11b, CD16, CD15, CD56, CD10, CD71, CD235a, CD36, CD7, CD2, CD5, and CD19. Cases were placed into "positive", "intermediate"and "negative"flow cytometric categories based upon numerical and antigenic aberrancies observed on blasts and myelomonocytic cells. Flow results were correlated with morphological diagnosis and cytogenetics. *Results.* Of the 34 cases, 22 (64.71%) had MDS by morphology while 11 (32.3%) exhibited no myelodysplasia. The remaining single case had dysplastic megakaryocytes only on BM biopsy. Of the 22 cases with MDS (8 RCUD, 7 RCMD, 1 RAEB-1, 4 RAEB-2 and 2 MDS-U), 19 (86.36%), 3(13.64%), and 0 (0.0%) cases were FCM positive, intermediate and negative, respectively. The patient with dysplastic megakaryocytes only, had abnormal flow pattern but responded to hematinics and hence was considered false positive for flow. The most common abnormality in the blastic population was abnormal expression of lymphoid antigens (CD2, CD5, CD7, CD19 or CD56) (n=12) while the commonest abnormalities in the maturation pattern were discernible with the following antibody combinations: CD34/CD38 (16/22; 72.7%), CD16/HLA-DR (13/22; 59.1%), CD13/HLA-DR (13/22; 59.1%), CD11b/CD15 (13/22; 59.1%) and CD13/CD16 (13/22; 59.1%). The overall sensitivity was 91.67% and overall specificity 86.36% and 100%, respectively, depending on whether the intermediate category was excluded or included as an indicator of MDS. In all patients with low grade MDS (n=17), PNH test was negative and therapeutic trial with vitamin B12 and folic acid had failed. Serum iron studies, serum vitamin B12 and folate levels were normal. Cytogenetics was normal in 21/22 (95.45%) and trisomy 8 was found in one patient. *Conclusion.* FCM is a very sensitive and useful tool for diagnosis of MDS. Abnormalities in blast segment and the maturation pattern of myeloid and monocytic cells of the BM are very informative. The small but significant number of cases that give false positive result, warrant analysis of normal controls and cases with secondary dysplasia using multicolor immunophenotyping.

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EVALUATION OF GENE METHYLATION STATUS AND FOLATE METABOLISM IN PATIENTS WITH MYELODYSPLASTIC SYNDROME

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Aberrant methylation patterns are common mechanisms in human neoplasia, especially in hematopoietic neoplasms. Many genes may be involved; cell cycle regulators, as the tumor suppressor gene p15, are frequently inactivated in MDS by aberrant methylation and have been correlated with the risk of evolution toward AML and poor prognosis. Several studies suggest that folate intake/status modulates DNA methylation. Functional polymorphisms in key genes may also influence DNA methylation namely those related with MTHFR. A common MTHFR gene polymorphism involving a C-T transition at codon 677 is associated with reduced methylation of CpG DNA in human lymphocytes under conditions of low folate status. Less is known about the A1298C polymorphism, but most studies have failed to show a relationship between this polymorphism and aberrant methylation. The aims of this study are the evaluation of the methylation patterns of genes involved in MDS, p15/p16, and its relationship with folate/vitamin B12 levels. We hope to contribute for the identification of molecular risk markers: the role of epigenetic gene modulation and its interference with prognosis, evolution to AML and therapeutic approach in MDS patients. We examined the methylation status of the cell cycle regulators p15/p16 in CD34 bone marrow cells populations collected at diagnosis from 26 patients with MDS and in 8 non-malignant controls(Immune Thrombocytopenic Purpura,ITP), using a methylation specific-PCR. We also evaluated the folate/vitamin B12 serum levels, by a commercial radioimmunoassay kit, and the MTHFR polymorphisms, C677T and A1298C, by PCR-RFLP, in patients and in 289 healthy controls. The median SMD patients age is 74 years(33-84), gender M/F=14/12, the distribution according WHO-subtypes is: RCMD(n=9), RA(n=5), RAEB-1(n=3), RAEB-2(n=5), CMML(n=3), 5q-syndrome(n=1) and according IPSS is: low(n=7), intermediate-1(n=13) and intermediate-2(n=6). Seven patients (6-RAEB and 1-CMML,IPSS intermediate-1 and -2) evolve to AML, with a median follow-up of 28 months(16-71). Our preliminary results show that 74% of cases of MDS have at least one methylated gene. p15 methylation occurred in 39% MDS patients, while p16 methylation occurred in 37%. p15 methylation was present in all the RA and 5q- syndrome patients and in 25% of patients with the RCDM subtype. On the other hand, p16 methylation was observed in all subtypes expect RAEB-2 and CMML (75%-RA and RCDM;100%-5q- syndrome and RAEB-1). Only 2 of the 7 patients who evolve to AML show aberrant methylation. The levels of folate/B12 seem to influence methylation, since low levels are associated with methylation, especially of p16. Twenty patients, mainly RCDM, have the polymorphism C677T, and this may be a risk factor for the disease (OR-3,982). However, the polymorphism A1298C also predominates in this population. This study suggest that p15 and p16 seem to be an event in the MDS development and serum folate/vitamin B12 low concentrations might be associated with the risk of promoter methylation in tumor-specific genes, especially p16 gene. On the other hand, in MDS patients, the MTHFR polymorphisms CT(C677T) and AC(A1298C) predominate, being the first one a risk factor for this disease. Surprisingly, in the majority of patients who evolve to AML, we didn't found aberrant gene methylation.

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THROMBOCYTOPENIA IN MYELODYSPLASTIC SYNDROME BASED ON THE DATA OF A SINGLE-CENTER ROMANIAN REGISTRY

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Introduction. Thrombocytopenia is a challenging finding in myelodysplastic syndrome (MDS) patients because, when severe, it is an important cause of death by life-threatening bleedings, and also it is difficult to manage because no approved specific pharmacologic therapy exists yet. The interest in MDS-related thrombocytopenia increased recently due to the promising results obtained with some specific drugs. *Material and Methods.* The patients recruited between 1982 and 2004 constituted the database of the MDS Registry comprising 403 primary adult cases. The registration form was kindly provided by the MDS Foundation (USA) and the diagnosis of MDS was based on well-accepted FAB minimal diagnosis criteria. Thrombocytopenia was defined as a platelet count ≤100,000/mcL and was divided in three subgroups of severity: mild (100,000-51,000), moderate (50,000-21,000) and severe (≤20,000). The parameters age, sex, rural/urban location, temporal trend and distribution by FAB subtypes were analysed comparatively in thrombopenic and non-thrombopenic MDS cases. The frequency, type and severity of hemorrhagic complications and of their implications on survival and clinical practice are also analyzed. *Results.* A retrospective review of thrombopenic patients at referral identified 182 cases (45%). The isolated thrombopenia represented 1% in the MDS registry and 2% in the thrombopenic cohort. There were 43% cases in the mild, 34% cases in the moderate and 23% cases in the severe group with a median platelet count of 77,000/mcl, 39,000/mcl and 14,000/mcl, respectively, with no differences among the age subgroups. No signif-

icant difference between the thrombopenic and non-thrombopenic cases concerning the epidemiological characteristics was found. The temporal trend of the thrombopenic cases was significantly higher in the group ≥60 years compared to ≤50 years, similarly with the trend found in the whole cohort of MDS patients of our registry. A significant predominance of the thrombopenic cases in the high-grade FAB subtypes was found. *Conclusions.* To our knowledge, this is the first epidemiological study on the MDS patients with thrombocytopenia in Europe and the presented findings are close to those reported in a similar study by the MD Anderson Cancer Center (USA) in 2007. However, the percentage of thrombopenic MDS patients appears significantly lower in the present study, reflecting the inherent difference in referral between a hematooncological center with a MDS specialized unit and a large but ordinary hematological department where bone marrow transplantation was not available.

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MNSOD ALA(16)VAL POLYMORFISM AS A RISK MARKER FOR MYELODYSPLASTIC SYNDROME?

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Myelodysplastic syndrome (MDS) is a heterogeneous stem cell bone marrow disorder characterized by the underproduction of one or more blood cells types, due to haematopoiesis dysfunction, and higher risk of leukemic transformation. The aetiology and pathogenesis of MDS remain poorly characterized and may arise *de novo* or be secondary to treatment with chemotherapy or radiation therapy. Indirect evidences suggest a role for oxidative stress (OS) in MDS aetiology and pathogenesis. OS, resulting from an imbalance between Reactive Oxygen Species (ROS) production and antioxidant defences, contributes to cell damage, apoptosis and ineffective haematopoiesis. A genetic polymorphism in the mitochondria targeting sequence of the MnSOD gene, resulting in a substitution of valine (Val) to alanine (Ala) at amino acid 16, leads to a defective localization of the antioxidant enzyme to the inner mitochondrial membrane. This polymorphism has been associated with an increase in breast, prostate and colorectal carcinoma cancer risk. However scanty studies are done in haematological neoplasias, namely in MDS. In the present study we set to investigate the influence of Val16Ala MnSOD polymorphism as a risk factor of MDS development and as a prognostic risk marker in MDS patients. For this purpose we analyzed MnSOD polymorphisms by PCR followed by restriction digestion in 33 MDS patients and 62 controls from the same geographical area, ethnic background and approximately similar age. The patient group median age was 79 years (33-84), gender M/F=18/15, WHO subtypes: RCMD (n=9), RA (n=9), RAEB-1 (n=3), RAEB-2 (n=7), 5q- syndrome (n=2) and IPSS: low (n=8), intermediate-1 (n=14) and intermediate-2 (n=6). Allele frequencies were determined by direct count of the alleles. Departures from Hardy-Weinberg equilibrium and differences between groups were evaluated by the chi-square test. ORs and 95% CIs were determined by using Fisher's exact Test and Kaplan-Meier survival analysis were used to investigate the prognostic importance of MnSOD polymorphism. Our preliminary results show a higher C allelic frequency in MDS patients (75%) while controls present a higher T allelic frequency (55%). In MDS patients the frequency of TT, CT and CC genotype was 6%, 54% and 40%, respectively, Besides that, individuals with CC genotype have an increase risk for MDS about 3,014-fold (CI95% 1,159-7,834; P=0,0269). On the other hand, the TT genotype might be a protective factor (OR=0,2022; CI95% 0,04317-0,9466; P=0,0464). Our results also show that MnSOD genetic polymorphism might be related with MDS subtype and prognosis, since we observed differences in MDS overall survival between genotypes (without significance). However, we are currently being tested this observation in a large cohort of samples. This study indicate that MnSOD polymorphisms may confer susceptibility to the development of MDS, since individuals with MnSOD CC genotype have an increase risk 3-fold, and may also be related with the subtype, prognostic and survival of MDS patients.

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ROLE OF WT1 IN JUVENILE MYELOMONOCYTIC LEUKEMIA

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Background. Approximately 75% of patients with juvenile myelomonocytic leukemia (JMML) harbour mutations in PTPN11, NF1 and RAS genes. Recently mutations in c-CBL were identified playing a role in the pathogenesis of JMML. Still, in about 15% of the cases no mutations are found. In adult patients with acute leukemia and MDS increased levels of WT1 expression are reported. In these patients determination of WT1 expression is considered to be a suitable technique for the monitoring of minimal residual disease. Furthermore, WT1 mutations predict poor outcome in adult and childhood acute myeloid leukemia (AML). In JMML WT1 expression levels in bone marrow have been reported to be significantly higher than in normal bone marrow. *Aims.* To investigate if the higher WT1 expression is caused by mutations in the WT1 gene and whether they play a role in the hyperproliferation in JMML. *Methods.* In 48 JMML patients screening for mutations in the whole WT1 gene was performed (exon 1-10) on DNA by PCR and direct sequencing. Quantitative expression analysis of WT1 was performed by qRT-PCR. *Results.* In only one patient a mutation was found in exon 1 of the WT1 gene. Mean expression levels were higher as compared to normal bone marrow, but lower as compared to acute myeloid leukemia. Expression levels were not correlated to mutation status, white blood cell count, blast count or absolute monocyte count. *Conclusion.* In JMML patients the expression of WT1 is higher as compared to normal bone marrow. This higher expression is not caused by mutations in the WT1 gene.

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SYSTEMIC AND IMMUNE MANIFESTATIONS IN MYELODYSPLASIA. A MULTICENTRIC RETROSPECTIVE STUDY.

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The presence of systemic and/or immune manifestations in myelodysplasia is classical, even thought rarely presenting as initial clinical event. *Purpose.* We present a multicentric retrospective study (2002-2009) with systemic and/or immune manifestations dominating the clinical presentation. *Patients et methods.* 46 patients with a formal diagnosis of myelodysplasia, presenting systemic and/or immune manifestations have been studied during a eight year period (2002-2009). The clinical picture in these cases consisted in transient fever, not related to infections. (17%), arthralgia or arthritis (12%), and especially cutaneous (purpura 25%, nodular 12%, papular 7%, and necrotic lesions 6%) related to cutaneous lymphocytary and/or leucocytoclasic vasculitis. In 8% of the cases this leucocytoclasic vasculitis was mainly present in muscular vessels, evoking panarteritis. Lung symptoms were occasional (4% of cases), the same as renal manifestations (4% of cases). Even though infrequently, some rare cases of systemic vasculitis have been reported in our series (one panarteritis 2%, one cas of Wegener granulomatosis, one cas of microscopic polyangeitis et one cas of Churg-Strauss syndrome). Immune anomalies were recorded in 29% of the cases (antinuclear antibodies 22%, ANCA 4%, rheumatoid factor 8%). A corticosteroid treatment was efficient in 92% of the cases. The presence of vasculitis during myelodysplasia has been reported in 63% of the cases, and represented a marker of acutisation (relapse) in 49% of the cases. A vasculitis initial clinical presentation has been found in myelodysplasia in 37% of the cases in our series (and preceded myelodysplasia with a mean period of 6 months). *Discussion and conclusion.* The association between myelodysplasic syndromes with systemic and immune manifestations seems to not be occasional and suggest the eventuality of a potential common primary immune disorder.

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HEMATOLOGIC RESPONSE TO AN ALTERNATIVE DOSING SCHEDULE OF AZACITIDINE IN HIGH-RISK MYELODYSPLASTIC SYNDROMES

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Background. Azacitidine (AZA) has proven effective in Myelodysplastic Syndromes (MDS), as it has been shown to induce: a) significant reduction of transfusion dependence; b) decreased risk of evolution into acute myeloid leukemia (AML); c) improvement of quality of life; d) significant increase of overall survival, as compared to conventional care regimens, in high risk patients (pts.) (Silverman LR, J Clin Oncol 2002; Fenaux P, Lancet Oncol 2009). The currently approved AZA regimen is 75 mg/sqm/die subcutaneously (SC) or intravenously (IV) for 7 days every 28 days. Recently 3 different AZA dosing regimens, which avoid week-end dosing, have shown to induce therapeutic responses consistent with the currently approved schedule (Lyons RM, J Clin Oncol 2009). In particular, one of them, i.e. the AZA 5-2-5 regimen (50 mg/m^2/d subcutaneously for 5 days, followed by 2 days no treatment, then 50 mg/m^2/d for 5 days) allows the administration of a nearly equivalent monthly total dose of AZA, saving 4 vials/month (28% of the actual cost). Moreover, some data suggest the possibility that prolonged exposure to lower doses of AZA may increase the response rate (Gore SD, Cancer Res 2006). However, the community-based study of Lyons mainly involved lower-risk MDS pts. *Aims.* These data prompted us to investigate the therapeutic effect of the more convenient and less expensive AZA 5-2-5 regimen in higher-risk MDS patients (i.e.: IPSS risk: high or intermediate-2). *Methods.* From December 2007, in our Institution, 10 consecutive MDS pts. (6 males), with a median age of 69 (52-79) yrs, were treated with the AZA 5-2-5 regimen. IPSS risk was high (2 pts) or intermediate-2 (8 pts). IPSS-based cytogenetic risk was: high (2 pts), intermediate (3 pts), low (5 pts). *Results.* The pts received a median number of 7 (3-10) AZA cycles. 7 pts. (70%) showed a favourable response, following IWG criteria (Cheson BD, Blood 2006): 1 Complete Remission (CR), and 6 Hematologic Improvement (HI). Among them, erythroid response was observed in 2 pts, platelet response in 5 pts and neutrophil response in 3 pts. 1st response occurred after a median of 3 (3-6) cycles. The 3 non-responder pts maintained a stable disease (SD) during treatment. 2 of them received at least 6 cycles of AZA, 1 pt stopped treatment after the 4th course because of worsening of clinical condition. 2/3 non-responder pts died, 1 following evolution into AML, 1 because of infection. 1 responder pt stopped treatment after the 8th cycle because of disease progression (AML), and is still alive. Summary/*Conclusions.* Our data seem to confirm, in a population of higher risk MDS pts, the favourable results obtained by Lyon with the 5+2+5 AZA regimen in lower risk pts. These results need to be supported by larger studies, and the effect of this alternative dosing schedule on survival also needs to be assessed.

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TREATMENT WITH AZACITIDINE IN THERAPY-INDUCED MSD. PRELIMINARY DATA FROM THE SPANISH AZACITIDINE COMPASSIONATE USE REGISTRY

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Background. Therapy-related Myelodysplastic Syndromes/Acute Myeloid Leukemias (t-MDS/AML) are one of the most compelling long term adverse events occurring in cancer survivors treated with chemoradiotherapy regimes. Beside several well-described genetic lesions, a growing amount of data suggests that abnormalities in DNA methylation profile contribute to multistep secondary leukemogenesis. Cytotoxic drugs and radiation have been shown to affect tissue DNA methylation profile. In general, patients with therapy-induced MDS have a poorer prognosis then patients with *de novo* MDS. Azacitidine (AZA) is a hypomethylating agent recently approved in Europe for the treatment of MDS. AZA was available in Spain under compassionate use before its commercial approval in May 2009. *Material and Methods.* We present the preliminary analysis of the clinical data from a longitudinal, multicenter Spanish patient registry. Data on the disease course and management of patients with MDS treated with AZA under compassionate use conditions were retrospectively collected from community-based hematology clinics. As of Febrary 1, 2010, 30 patients with intermediate-2/high IPSS-risk therapy-induced MDS diagnosed according to WHO criteria had been included. *Results.* At baseline the median age was 76 years, the male/female ratio was 13/17 with an ECOG performance status of 0-1 60%. The most frequent initial dose of AZA applied was 75 mg/m^2 AZA was administered mostly subcutaneously (80%). The mean number of cycles administered was 6. The overall treatment response was 46,6% (International Working Group 2006 criteria): 16% complete response, 3,3% complete bone marrow response, 6,6% partial response and 20% hematological response. In addition, 23,3% achieved stable disease. AZA was generally well tolerated. The grade 3/4 adverse events documented in these patients, regardless of their relationship to active treatment, were neutropenia (26%), thrombocytopenia (33%), anemia (20%), febrile neutropenia (3%), rash (6%), colicky lumbar pain (3%) and subcutaneos zone reaction (6%).Similar response rates were observed in patients with MDS secondary that in primary ones. *Conclusion.* Our results demonstrate that in a community-based setting, patients with intermediate-2/high-risk therapy-induced MDS respond to treatment with AZA. These favorable results suggest that patients with secondary MDS may benefit of 5-AZA. More data coming from this registry and from the clinical trials are awaited.

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PRELIMINARY SAFETY ANALYSIS OF AN EXPANDED ACCESS PROGRAM FOR DECITABINE IN SUBJECTS WITH MYELODYSPLASTIC SYNDROME (MDS)

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Background. Decitabine is a hypomethylating agent, approved by the FDA for treating MDS. In 2008 an expanded access program was established in Brazil to make decitabine available to subjects with MDS. *Aims.* To present the results of an interim analysis, regarding the patient's baseline characteristics and safety profile. *Methods.* Between August 2008 and June 2009, 30 Brazilian patients diagnosed with *de novo* MDS were enrolled for treatment with decitabine in 9 different centers. The decitabine schedule used in all the subjects was 20 mg/m^2/day for 5 days, in a four-week cycle for four cycles. To associate some baseline characteristics to the occurrence of adverse events during the course of treatment we underwent an analysis using the chi-square test. *Results.* The median age was 65.5 (range 30-80), most of the patients were men (60%) and had a good Performance Status (ECOG 0+1: 86.7%, ECOG 2: 13.3%). Regarding IPSS, 19 patients (63.3%) were Int-1, 7 were Int-2 (23.3%) and 4 (13.3%) were High risk. All the patients had anemia, 63.4% were neutropenic and 60% were thrombocytopenic before starting the treatment. Regarding the cytogenetics, most of the patients (66.7%) had a good prognosis karyotype. Eighty percent were at transfusion support, mostly with RBC transfusions. The most frequent hematologic adverse events related to decitabine were: neutropenia (63.3%), thrombocytopenia (53.3%) and anemia (36.7%). Febrile neutropenia related to the treatment occurred in 43.3% of the patients and infections were detected in 12 (40%). Dose reductions and dose delays were performed mostly after the first cycle (12% and 71.8%, respectively), mostly due to hematologic toxicity. The transfusion need had a small increase after the first cycle (80-86.7%), but decreased progres-

sively until the fourth cycle (45%). During the treatment with decitabine, 80% of the patients required antibiotics, either prophylactic or therapeutic. In an association analysis, it was observed that the patients with ECOG Performance Status 2 were at a higher risk of developing infection (P=0.016), hemorrhage (P=0.031) and constipation (P=0.031). No statistically significant difference was found among the FAB classification and IPSS score groups regarding the occurrence of adverse events. We also observed that patients from the age group 60-75 years had a higher percentage of neutropenia than the group older than 75 years (P=0.018). In the sample of 30 patients, 9 (30%) died most deaths related to infections, and 3% discontinued the study medication during the four cycles. *Conclusion.* In this sample, the FAB classification and IPSS had no statistically significance in the risk of development infection, hemorrhage or constipation. Patients with ECOG 2 were at higher risk of developing these adverse events.

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DECREASED EXPRESSION OF RIZ1 AND ITS CORRELATION WITH RISK STRATIFICATION IN MDS PATIENTS

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Background. RIZ1, the retinoblastoma protein-interacting zinc finger gene, is now considered to be a candidate tumor suppressor in many types of human cancers. Expression of RIZ1 is reduced in a variety of human solid tumors including breast cancer, liver cancer, colon cancer, neuroblastoma, melanoma, lung cancer, and osteosarcoma. Suppression of RIZ1 expression is also found in a few of neoplastic hematologic disorders such as acute myeloid leukemia (AML), chronic myeloid leukemia (CML) and lymphomas, indicating that downregulated expression of RIZ1 may play an important role in leukemogenesis and disease progression. However, alteration of RIZ1 gene expression in myelodysplastic syndrome (MDS) has not been reported to date. *Aims.* To investigate the expression of RIZ1 and its correlation to development, disease progression and risk stratification in human MDS. *Methods.* We analyzed the expression of RIZ1 by quantitative real-time reverse-transcription polymerase chain reaction assay in 22 untreated MDS patients and in 6 patients with nonneoplastic hematologic diseases as control. Results were expressed as a ratio between the RIZ1 mRNA expressed in MDS and normal bone marrow cells. Values that differed from normal cell values by 2 standard deviation (SD) units were considered significant. *Results.* The expression of RIZ1 was significantly decreased in MDS group (0.59±0.27 *vs.* 1.00±0.19, P<0.05, compared with control group) in 15 of 21 (71%) patients. When compared with noncancerous patients, MDS patients of RA subtype (0.84±0.36) owned the statistically same expression of RIZ1 (P>0.05), but patients of RCMD (0.29±0.05), RAEB-1 (0.64±0.15) and RAEB-2 (0.31±0.09) subtypes showed a significantly decreased expression of RIZ1 (P<0.05). Likewise, RIZ1 mRNA level decreased obviously in high risk group (including RAEB-1 and RAEB-2 subtypes; 0.49±0.18, P<0.05), but had no statistical change in low risk group (including RA, RCMD and 5q- subtypes; 0.70±0.35, P>0.05). The RIZ1 expression of high risk group tended to be lower than that of low risk group, but the difference was not statistically significant. *Summary.* Decreased gene expression of RIZ1 is very common in human MDS and its level alters among WHO subtypes or risk groups. The expression of RIZ1 may contribute to the evaluation of development, progression and risk-stratification in MDS.

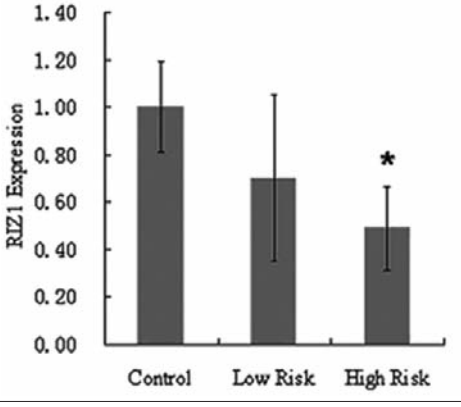

Figure. RIZ1 expression in MDS patients. *P<0.05.

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IMPACT ON THE SURVIVAL OF PROLIFERATIVE TYPE MYELOMONOCYTIC LEUKEMIA DIAGNOSED PATIENTS

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Background. Chronic myelomonocytic leukemia (CMML) is characterized by its extensive heterogeneity clinical and evolutionary, behaving as a mielosdisplastic syndrome (MDS) with defective and ineffective clonal cell proliferation or features of mieloprolyferative chronic disorders (cMPD) with Leukocytosis, monocytosis, organ involvement (eg.splenomegaly) and hypermetabolism. The scores used in the MDS are not reproducible to predict its evolution, and there are no studies that indicate the start of an active treatment based on prognostic factors. *Aims.* The study aimed to identify the variables to the diagnosis of cmml with impact on the risk of transformation in acute myeloid leukemia (AML) and overall survival (OS) and propose a new prognostic score index to identify high-risk patients and treated early. *Patients and Methods.* They have been revised as retrospective features 44 cmml criteria the FAB and between the years 1978-2010 WHO diagnosed patients. The median age was 80 years old and 37 were men (84,1%). According to the criteria of the FAB 26 patients have the MDS and 18 cMPD and according to the WHO clasification 86.4% (N = 38) were CMML-1 (blasts <10% in bone marrow and <5% in blood) y 13,6% (n=6) were cmml-2. They valued the alterations in the karyotype of 38 patients (altered 12) and find the presence of organomegaly in 29.5% (n = 13) patients. With a point cut of Hb 10 g/dL, 15 patients showed lower figures and the number of LDH > 400 U/L observed in 69,2% (N = 27) of patients. β-2 microglobulin (>4mg/L) was studied in 27 patients and were abnormal in 47% of patients. Having identified these variables from poor prognosis, we evaluated the OS curves built with KAPLAN-MEIER method. *Results.* In the sample we have evaluated the wrong variables forecast and its impact on survival. The organomegaly was present at 61.5% of the cMPD-CMML (P=0.07), 66.7% of patients with abnormal karyotype was proliferative type and the LDH > 400 U/L was mostly in these patients. 27 Patients studied β-2 microglobulin 69,2% (P=0, 35) were myeloproliferative-CMML and moving those variables to survival curves,patients with cMPD-CMML have a 4.7 months *vs.* 28.3 survival in patients that no presented. It was noted the value separately as well as in conjunction with significant decrease in survival. *Conclusions.* The results of our study confirm the poor prognosis of patients of CMML associated with other variables such as alterations in the karyotype, proliferative type, Hb<10 g/dL, levels of β-2 microglobulin >4 mg/L. A clinical trial with new drugs is needed to assess the effectiveness of early treatment in high-risk patients.

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FREQUENCY OF H63D MUTATION IN THE HFE GENE IN ADULT PATIENTS WITH MYELODYSPLASTIC SYNDROMES

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Background. The Hereditary hemochromatosis gene (HFE) plays a pivotal role in iron homeostasis. An increased incidence of HFE gene mutations has been described in hematologic malignancies, including acute leukemias and lymphoproliferative disorders. On the contrary, few data are available in patients with myelodysplastic syndromes (MDS), in which HFE mutations could have major clinical relevance, by interfering with iron overload due to transfusion dependence. Among general population, the allelic frequencies of C282Y and H63D, which is the more frequent mutations of HFE gene in Italy, are 0.037 and 0.16 in the Northern regions and 0.015 and 0.16 in the Southern regions. *Aims.* To investigate the allelic frequency of HFE gene mutations in 51 adult patients with MDS, diagnosed according to the current WHO criteria; 25 patients had refractory anemia (RA), 21 refractory anemia with blast excess (RAEB1= 9, RAEB2= 12) and 5 as refractory cytopenia with multilineage dysplasia (RCMD); MDS data were compared to those deriving from 230 healthy blood donors. In addition, data were also compared with those from a series of 107 patients with acute myeloid leukemia (AML) other than acute promyelocytic leukemia. We did specifically focus on H63D mutation, which either in the study group

or in the controls was the most frequent. *Methods.* HFE genotyping was performed by a polymerase chain reaction (PCR) method using sequence-specific-primers, and the products were analyzed on agarose gel and by Reverse Dot Blot. Statistical differences between the prevalence of HFE genotypes in the patients and controls as well as between different diseases and subtype of disease were assessed using the chi square test. P values were considered as statistically significant at a value <0.05. In addition, for any difference Odd's ratio and confidence intervals (CI) were also calculated. *Results.* The median age was 75 years for MDS patients (range 24-92), 40 years for blood healthy donors (19-55) and 55 years for AML patients (14-90). The allelic frequency of H63D mutation was 21 % in MDS patients and 25% in the healthy controls (P=0.58; odds ratio = 1.20; 95% CI=0.77 to 1.93). Furthermore, no significant difference was found between MDS and AML patients (21% *vs.* 29%, P=0.20; odds ratio = 1.37; 95% CI = 1.05 to 5.36). Finally, no statistically significant difference was found among the two main MDS subtypes (16% for RA *vs.* 24 % RAEB, p: 0.43; odds ratio = 1.20; 95% CI = 0.78 to 4.35). *Conclusions.* Our data demonstrate the absence of correlation between the presence of H63D mutation and the occurrence of MDS as opposed to normal population. In addition, no difference exists in the allelic frequency of the mutation between MDS and AML as well as between early and late MDS.

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ROLE OF ANTIAPOPTOTIC AND PROANGIOGENIC CYTOKINES IN THE COURSE OF DIFFERENT SUBTYPES OF MYELODYSPLASTIC SYNDROME

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Background. Significant importance in pathogenesis of myelodysplastic syndrome (MDS) belongs to the process of angiogenesis stimulated mainly by vascular endothelial growth factor (VEGF), however, some other cytokines, particularly transforming growth factor-β (TGF-β), also show certain angiogenic activity. Aim of this study was to determine the role of antiapoptotic and proangiogenic cytokines in the course of certain MDS subtypes with different levels of malignancy (refractory anemia (RA), refractory anemia with excess of blasts (RAEB)). *Methods.* Twenty one patients with MDS RA (mean age of 49,10±3,80) and 6 patients with MDS RAEB (mean age of 69,67±2,68) were investigated. The control group included 27 healthy volunteers with their age ranging from 20 to 54. TGF-β and VEGF levels were determined in blood serum by immune-enzyme analysis using the standard BIOSOURSE kits, USA. *Results.* Level of the antiapoptotic cytokine TGF-β was higher in patients with MDS RAEB. Three of RA patients had TGF-β levels higher, than the control group and other 18 showed the same levels as the healthy volunteers or even lower. Serum level of the proangiogenic cytokine VEGF was quite variable in patients with RA ranging from 15,43 to 183,21 pg/mL. In 5 RA patients it was considerably lower than the control value (114,44±11,08 pg/mL) and only in one case its level was twice as high as the controls. Among 6 patients with RAEB three had serum VEGF levels 2,5 times higher than the controls, whereas in other three patients its values were 4 times lower than in the control group. Blood and bone marrow investigations of the RAEB patients showing higher VEGF levels revealed prevalence of proliferative activity (increased level of peripheral blood leukocytes, hypercellular bone marrow); at the same time patients with lower levels of VEGF showed peripheral blood leucopenia and bone marrow hypocellularity. The course of MDS was quite stable in the latter group of patients; none of them developed transformation to acute leukemia during one year, despite persistent anemia, thrombocytopenia, increased blast counts. On the contrary, two of the three patients showing high VEGF levels progressed to acute leukemia 8 and 10 months after the first diagnosis respectively. There was a tendency observed that patients with low VEGF levels showed also low values of TGF-β, whereas patients with progressive disease had levels of both cytokines increased. *Conclusions.* Analysis of data obtained show that certain role in development of leukemic transformation of MDS belongs not only to the pro- and antiapoptotic cytokines but also to the process of angiogenesis. Relatively stable disease without progression in patients with MDS RA may result from the low TGF-β and VEGF production and increased apoptosis of hemopoietic cells. Elevation of TGF-β causes increase of proliferative potential and apoptosis inhibition, which along with activated angiogenesis induces disease transformation. Impaired angiogenesis (probably due to fibrotic changes in the bone marrow) even in condition of elevated TGF-β may contribute to disease stabilization. This data may explain certain success achieved by administration of antiangiogenic and proapoptotic medications in MDS patients.

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IMMUNOBIOLOGY AND IMMUNOPHENOTYPE OF PERIPHERAL BLOOD MONOCYTES IN CHRONIC MYELOMONOCYTIC LEUKAEMIA

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Chronic Myelomonocytic Leukemia (CMML) is a clonal disorder of the bone marrow resulting in monocytosis coupled with dysplasia in one or more myeloid cell lineage. Although classified as a separate disease CMML shows similarities with both myelodysplastic syndrome (MDS) and myeloproliferative disorder (MPD). Previous studies have shown excessive apoptosis in the bone marrow of MDS patients in addition to increased levels of Bcl-2 whilst in MPD, proliferation predominates. Using flow cytometric techniques peripheral blood $CD14^+$ monocytes were analysed for markers of proliferation (Ki-67) and apoptosis (Annexin V/Propidium Iodide) in addition to intracellular apoptosis regulatory proteins Bcl-2 and BID. Investigations were also carried out into the cell surface immunophenotype of CMML circulating monocytes. A tendency towards proliferation over apoptosis was observed in CMML compared with controls. Significant findings were reduced expression of both Bcl-2 and BID (P=0.015 and P=0.009 respectively), Bcl-2 expression correlating with haemoglobin levels and white blood cell count. Further correlations were seen between BID and both haemoglobin level and bone marrow blast cell count. Significant increased expression of CD56 on $CD14^+$ monocytes was observed along with decreased expression of CD3 and CD4 on lymphocytes. Correlations were found between CD56 and both Bcl-2 expression and bone marrow blast count. These findings, although preliminary, show that CMML may be more closely related to MPD, rather than MDS. Also a distinct lack of BID contributes to the pathogenesis of CMML and that CD56 expression may be used as a favourable prognostic marker.

1400

CIRCULATING MALIGNANT CLONAL CELLS IN PERIPHERAL BLOOD IN MDS: VERIFICATION USING INTERPHASE FLUORESCENT *IN SITU* HYBRIDIZATION AND ITS CORRELATION WITH BONE MARROW CLONAL CELLS

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Background and Aims. Bone marrow (BM) cells are believed to be the source of neoplasm in the myelodysplastic syndromes (MDS). We investigated whether the clonal proliferating pattern of peripheral blood truly reflects that of bone marrow. *Methods.* Interphase fluorescence *in situ* hybridization (iFISH) was performed on peripheral blood mononuclear cells from 9 MDS patients who had showed clonal abnormalities in the bone marrow at the initial diagnosis. Collection time of the peripheral blood sample, bone marrow diagnosis, type of clonal abnormality detected in bone marrow at the initial diagnosis, and the quantity of the clonal abnormality are as follows: patient 1 - initial diagnosis, RCMD, del(5q), 86.0%; patient 2 - initial diagnosis, RAEB-1, del(5q), 80.0%; patient 3 - initial diagnosis, RCUD (RN), tetrasomy 1q, 66.5%; patient 4 - follow up without stem cell transplantation (SCT), RAEB-2 progressed from RAEB-1, monosomy 7, 90.5%; patient 5 - follow up without stem cell transplantation SCT, RCUD (RA) sustained from RCUD (RA), del(5q), 45.0%; patient 6 - follow up after SCT, no engraftment after SCT in RCUD (RA), trisomy 1q, 5.0%; patient 7 - follow up after SCT, complete response after SCT in RAEB-1, trisomy 1q, 31.0%; patient 8 - follow up after SCT, complete response after SCT in RAEB-2, trisomy 1q, 64.5%; patient 9 - follow up after SCT without follow up bone marrow study (peripheral blood study only), RCUD (RA) at the initial diagnosis, trisomy 8, 69.0%. Probes (Vysis Inc.) were used to detect 5q, 7q, 8 and 1q abnormalities. The results were compared with iFISH results of bone marrow at the initial diagnosis and at the follow up. *Results.* The same types of clonal abnormalities were detected in similar quantity in the initial bone marrow, the follow-up bone marrow and the peripheral blood mononuclear cells in patients 1, 2, 3, 4 and 5. Patients 6, 7 and 8 showed no clonal abnormalities in peripheral blood and bone marrow at the follow up. Patient 9 showed the same clonal abnormality (trisomy 8) in peripheral

blood at the follow up as in the bone marrow at the initial diagnosis in a reduced dose (69.0% *vs.* 2.5%). *Conclusions.* We concluded that the types and the quantities of clonal abnormalities in the peripheral blood mononuclear cells well reflect those of bone marrow. Our results suggest the potential use of peripheral blood cells in the disease monitoring of the MDS patients without invasive bone marrow study.

Table. Profiles of the patients and the samples.

Patient No.	Initial BM/Follow up BM	SCT*	PB collection time	Initial BM FISH/ Follow up BM FISH/ PB FISH
1	RCMD/-	No	Initial Dx.	deletion 5q (86.0%/-[1]/75.5%)
2	RAEB-1/-	No	Initial Dx.	deletion 5q (80.0%/-/77.0%)
3	RCUD (RN)/-	No	Initial Dx.	tetrasomy 1q (66.5%/-/25.0%)
4	RAEB-1/RAEB-2	No	Follow up	monosomy 7 (90.5%/94.5%/82.5%)
5	RCUD (RA)/ RCUD (RA)	No	Follow up	deletion 5q (45.0%/47.5%/37.5%)
6	RCUD (RA)/No engraftment	Yes	Follow up	trisomy 1q (5.0%/0.3%/0.0%)
7	RAEB-1/Complete response	Yes	Follow up	trisomy 1q (31.0%/0.7%/0.0%)
8	RAEB-2/Complete response	Yes	Follow up	trisomy 1q (64.5%/-/0.0%)
9	RCUD (RA)/No follow up BM	Yes	Follow up	trisomy 8 (69.0%/-†/2.5%)

*SCT: Stem cell transplantation at least 14 days before collection of peripheral blood

[1]-: not tested

1401

CURRENT MANAGEMENT AND SURVIVAL OF IRISH PATIENTS WITH MYELODYSPLASTIC SYNDROME.

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Background. Myelodysplastic Syndromes (MDS) is commonly associated with a poor prognosis in high risk patients, and an indolent course in low risk patients. As a result, patients may not be eligible for active treatment. *Aims.* To examine the management strategies and survival of MDS in an Irish patient population. *Methods.* We prospectively collected data regarding management and survival for MDS patients from our own database (1999-2009) and those registered with the National MDS Registry. *Results.* Data is presented for 220 patients. Median age was 73 years (range 15-94 years). Where data is available regarding WHO categorisation, 153 (69%) patients had RA, RCMD, RARS or RCMD-RS and 67 (30%) had RAEB or CMML. 152 patients (69 %) required transfusion. 34 (15%) received transfusion therapy alone; only 87 received Erythropoietin (39%). Four patients received iron chelation therapy. Thirty seven (16.8%) patients were treated with GCSF. 25 (11%) patients received both Erythropoietin and GCSF therapy. Forty nine patients (22%) received low dose chemotherapy, 15 were treated with Azacytadine and 30 with Hydroxyurea - 4 patients received both. 4 were treated with Thalidomide and 6 received Lenalidomide. Twenty one patients received AML induction-type therapy and 8 patients remain alive. Eight patients were transplanted (one autograft and 7 allografts). Of the patients where follow-up information is available, 110 remain alive and 90 have died. The median survival for the entire group was 22 months, 25 months for the 135 patients with data from the low grade MDS and 17 months and for the 65 with data from high grade group. *Summary/Conclusions.* MDS remains a disease that is not always actively managed although management options have improved with the onset of new medications such as Azacytadine. 39 (17%) received no treatment either in the form of medication or transfusion. Less than half of transfusion-dependent patients receive Erythropoietin therapy and very few are treated with iron chelation therapy. A small number receive aggressive chemotherapy. Most patients with MDS die as a result of their disease, and long-term survival is rare in high-risk disease.

1402

EFFICACY AND TOLERABILITY OF 5-DAY AZACYTIDINE DOSE INTENSIFIED REGIMEN IN HIGHER RISK MYELODYSPLASTIC SYNDROMES

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Background. Azacitidine is a hypomethylating agent indicated for treatment of higher risk Myelodysplastic Syndromes (MDS). A recently published phase III trial demonstrated improved overall survival (OS) of MDS patients treated with azacitidine compared to those receiving conventional care regimens, thus establishing this treatment option as first line therapy in those patients for whom bone marrow transplantation is not an option. The recommended azacitidine dosing regimen is 75 mg/m^2/day subcutaneously for 7 days every 4 weeks. Three alternative dosing were recently compared, achieving similar results to the approved schedule. Limited resources and patient comfort would favour a five day regimen if this was equally effective. *Aims.* Evaluate the efficacy of a 5-day dose intensified azacitidine regimen in terms of transfusion independence (TI), overall response (OR), time to Acute Myeloid Leukemia (AML) transformation and tolerability in patients with higher risk MDS and AML with 20-30% blasts. *Methods.* Higher risk (International Prognostic Scoring System - IPSS intermediate-2 and high risk) MDS patients were treated with the 5-day intensified azacitidine schedule (100 mg/m^2/day subcutaneously for 5 days every 4 weeks) in our institution. OR, including complete response (CR), partial response (PR) and haematological improvement (HI), defined according to the 2000 International Working Group Criteria (IWG), were assessed by blood and bone marrow examination after the sixth cycle. Treatment cycles were repeated until toxicity or disease progression. *Results.* A total of 13 patients were treated: nine patients with refractory anaemia with excess blasts (RAEB), two with AML with 20-30% blasts and two with chronic myelomonocytic leukaemia (CMML). The average age was 71 years old (range 33-85) and 77% were men. Most patients were high risk according to IPSS scoring (77%). Azacytidine was used as first line therapy in 42%. An average of 7,5 cycles (1-18) per patient were administered. Four patients interrupted treatment following the first cycle, one due to disease progression and three due to patient refusal following admissions with pneumonias. The average follow-up period was 21 months. The overall response rate was of 53%, all of whom became TI, with average response duration of 11 months. Marrow responses were obtained in 2 patients (15%) (1 CR and 1 PR) both of which had good risk cytogenetics. During the follow-up period, nine patients died with average interval between diagnosis and death of 25 months. Six deaths were due disease progression whilst three were due to acute pneumonia in the absence of disease progression. Ten patients (77%) had Grade III haematological toxicity and three (23%) suffered Grade IV haematological toxicity. Grades I and II gastrointestinal and skin toxicity were recorded in all patients. *Conclusions.* The efficacy of Azacitidine in achieving TI and prolonging survival in MDS is well recognized. However, the recommended 7 day regimen is often difficult due to limited resources. In this small cohort a 5 day dose intensified azacitidine schedule was well tolerated and achieved similar response rates to those published. Larger studies would be necessary to validate this regimen.

1403

HIGH-RESOLUTION SNP ARRAYS AS AN ADDITIONAL TOOL TO SEARCH FOR GENETIC DEFECTS INVOLVED IN PROGRESSION FROM MYELODYSPLASTIC SINDROME TO ACUTE LEUKEMIA

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Myelodysplastic syndromes (MDS) often progress to acute myeloid leukemia (AML), but the molecular basis of this transformation are not fully known. In order to find clues to identify genes involved in progression, we studied a high-risk MDS patient with high resolution SNP arrays (molecular cytogenetics) at different stages of the disease. This technique can detect lesions between 1-10 Mb and map two different genetic alterations: copy number variants (CNVs) and copy neutral-loss of heterozygosity (CN-LOH). CNVs comprise submicroscopic variants of 1 kb or larger, which include deletions, duplications, insertions, inversions and traslocations not present in a reference genome while CN-LOHs represent extended tracts of homozygosity occuring without concurrent changes in the gene copy number (CN). We report of a 70y man presented with mild anemia, neutropenia and thrombocytopenia. A diagnosis of MDS AREB2, Intermediate-2 IPSS, was done on the basis of bone marrow (BM) analysis (trilineage displasia with 15% blasts) and cytogenetics (complex karyotype with 3 unrelated aberrations including -7). Therapy with rhuEPO was started with no benefit. Four months after diagnosis cytopenias worsened and BM blasts raised to 20%. RBC transfusions and therapy with 5-azacytidine (5-AZA) were started. After 2 cycles we observed a striking improvement of Hb and

PLT and, after 4 cycles, in WBC too. BM biopsy showed no blasts, karyotype was normal except 45X, -Y in 3/20 metaphases. After 11 cycles a decrease in PLT levels was noticed and 18% blasts reappeared in BM. The complex karyotype reappeared with additional chromosome abnormalities. 5-AZA was stopped and lenalidomide was started, with significant benefits already after the first cycle. However, after the 2nd cycle both WBC and PLT worsened again. Therapy was continued for 2 more cycles, when BM analysis (80% blasts) and cytogenetics (100% complex karyotype) were repeated leading to a diagnosis of overt AML secondary to MDS. The patient soon died for multiorgan failure due to disease progression. Compared to conventional cytogenetics, molecular cytogenetics identified a conversion of the partial trisomy to partial tetrasomy 8 that occurred during the progression of the disease due to the addition of an extra copy of the whole chromosome 8 and a CN-LOH on the q-arm of chromosome 11. SNP array analysis in different phases of the disease provided information also on the temporal order of appearance of the various abnormalities. Both partial chromosome 8 tetrasomy and 11q CN-LOH were late events during the progression of the disease, thus not to be considered primary genetic defects initiating MDS. We then searched for point mutations in 11q genes to pinpoint eventual homozygous mutations that could have played a role in the pathogenesis of MDS/AML and found a novel mutation in c-Cbl gene (K382E). The mutation appeared in a subclone at diagnosis and became predominant at late relapse. Serial application of conventional and novel molecular cytogenetics techniques provided new hints on the possible involvement of novel genes in the leukemogenic process. SNP arrays revealed not otherwise observed genetic defects and quantified the enrichment of cells bearing chromosomal abnormalities during disease progression.

1404

LENALIDOMIDE IN LOW RISK MYELODYSPLASTIC SYNDROME: SINGLE CENTRE EXPERIENCE

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Background. Low risk myelodysplastic syndrome (MDS) is characterized by transfusion dependent anemia and lower propensity to progression to acute myeloid leukemia. Lenalidomide, an immun-modulatory agent has been shown to be effective in treatment of low risk MDS associated with 5q del abnormality. Responses in transfusion-dependent non-5q del MDS appears to be significantly lower, with approximately 21% becoming transfusion independent. *Aims.* Prospective study to evaluate safety and efficacy of lenalidomide in the treatment of low risk myelodysplastic syndrome was carried out from July 2008 to Dec 2009. *Methods.* All the patients with low risk *de novo* MDS (IPSS low & int-1), with transfusion dependent anemia were included in study. Patients with a base line platelet count <50×10^9/L, absolute neutrophil count (ANC) <1×10^9/L were excluded. Lenalidomide starting dose -10mg/day with modification for cytopenias. Minimum period of study period was 4 months with the drug to be continued in the responding patients. All the patients were monitored for response (IWG criteria) and toxicity. Institutional ethics committee approval and patient's consent was taken before starting therapy. *Results.* Fifteen patients were prospectively enrolled for lenalidomide therapy, out of which 14 (Male: female: 6:8) have completed 4 months of therapy while one patient was lost to follow up. Mean age was 46.7years (27-72years). WHO types: RA-4, RARS-1, RCMD-3, RAEB1-2, MDSU-1, MDS with 5q del3 (RAEB2-1, RA-2). In twelve patients cytogenetics data was available (normal- 8, 5q del-3, 44xy with del2 & del18-1). Serum EPO level was more than 500 mIu/mL in all patients. Base line mean hemoglobin was 5.2gm% (3-6.7), ANC 2.8×10^9/L (1.4-5.4), platelet count 171×10^9/L (51-740). Five patients became transfusion independent (overall response 5/14 (35.7%)- two of the three (66%) patients with 5q del & three of the eleven patients without 5q del (27.7%) responded while 9 patients did not show any improvement. Median time to response was 8 weeks (4-12 weeks) with a mean rise in hemoglobin of 5.3gm%, and median follow up of 12 months (2-15 months). Early myelosuppression was the predominant drug toxicity, occurred in 11/14 (78.5) % of patients, however grade 3/4 neutropenia & thrombocytopenia occurred in 9/14 (56%) patients requiring temporary drug interruption. At the end of 4 months 9/14 patients were on 5 mg/day dose of lenalidomide. Two patients required hospitalization for neutropenic fever and platelet transfusion support. Renal and liver dysfunction was seen in none but one patient had skin allergic reaction requiring antihistaminics. All responding patients are still on treatment & transfusion independent (one-10 mg/day, three- 5 mg/day, one- 5 mg/alternate days). *Conclusion.* In our study lenalidomide was found to effective in low risk MDS with 35% response rate irrespective of cytogenetic abnormality. Predominant toxicity is myelosuppression necessitating frequent monitoring, dose modification & supportive treatment. A lower dose of 5 mg/day appears to be effective and better tolerated in low risk MDS patients. A further large multicentre trial of low dose lenalidomide is warranted to validate this approach.

1405

EFFICACY OF 5-AZACITIDINE IN THE TREATMENT OF HIGHER-RISK MYELODISPLASTIC SYNDROMES, ACUTE MYELOID LEUKEMIA AND CHRONIC MYELOMONOCYTIC LEUKEMIA: EXPERIENCE AT A SINGLE CENTER

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Introduction. The AZA-001 trial established 5-Azacitidine (5-AZA) as the first drug treatment to significantly extend overall survival (OS) in patients with myelodysplastic syndromes (MDS) compared with conventional care regimens (CCR) and showed a significantly haematological improvement (HI) of 49% of patients with 5-AZA *vs.* 29% with CCR. The role of 5-AZA as maintenance therapy is currently being investigated, after intensive chemotherapy, in patients with high risk MDS (HR-MDS) or acute myeloid leukemia (AML), not eligible for stem cell transplantation. *Aims.* 1- To assess the global haematological response (transfusion independence) and the tolerance with 5-AZA treatment in higher-risk MDS and CMML. 2- To assess the role of 5-AZA as maintenance therapy in patients with AML not eligible for stem cell transplantation. *Methods.* Patients have received 5-AZA at a starting dose of 75 mg/m^2 for five days of 28-day cycle. Table 1. *Results.* Between April , 2008, and February, 2010, 17 patients have been treated, with a median 10 months monitoring (3-23). We have defined haematological response as the achievement of transfusion independence and the maintenance of that independence. About 47% of patients have obtained haematological response with a median first response after 2 cycles (1 to 7) and highest response after 3 cycles (1 to 16). The most common side effects have been neutropenia (47%) and thrombocytopenia (30%). About 41% of patients have been hospitalized because of intravenous antimicrobial requiring infections and 23% have required G-CSF along the treatment. We have made subgroup analysis among patients treated previously with intensive chemotherapy. About 83% have obtained haematological response; toxicity has been comparable to the global analysis and only 2 of 6 have required intravenous antimicrobial treatment. Only one of these patients was transfusion dependent at the beginning of 5-AZA treatment. Death has occurred in 2 patients due to progression to acute myeloid leukemia, nevertheless, one of the patients has progressed 6 months after 5-AZA discontinuation (discontinued because non-related comorbidities: ischemic cardiopathy, temporal arteritis).

Table 1.

		AGE (Mean years)	TRANSFUSION DEPENDENCE BEFORE 5-AZA (Patients)	MEDIAN CYCLES
PATIENTS (TOTAL)	**17**	**71**	**7/17**	**4**
HR-MDS	3	68	2/3	3
CMML	6	71,5	2/6	4
AML	8	71	3/8	7
•Previous chemotherapy	**6**	**68,5**	**1/6**	**9**
•Not chemotherapy	2	76,5	2/2	4,5

Conclusions. The haematological responses obtained with 5-AZA treatment in our group of patients are comparable to AZA-001 trials results, with lower dose of 5-AZA (75 m/m^2/5 days of 28 days *vs.* 7 days of 28). In the previously intensive chemotherapy treated subgroup (initially poor prognosis subgroup not elegible for TPH), 5-AZA has been effective at achievement and keeping transfusion independence without an increase of toxicity. The time of monitoring is not enough to obtain conclusions about extend of overall survival, nevertheless, in our group of previously chemotherapy treated patients, about 66%

remain still alive with haematological response with a median of monitoring time of 9 months. These data suggest 5-AZA as a good maintenance therapy in these patients.

1406

ELEVATED FERRITIN VALUES ARE COMMON AT PRESENTATION IN IRISH PATIENTS WITH MYELODYSPLASTIC SYNDROME

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Background. Myelodysplastic syndromes (MDS) are clonal disorders of the marrow resulting in ineffective haematopoiesis, with premature intramedullary destruction of abnormal precursor cells and peripheral cytopenias. Morbidity and mortality in MDS results mainly from the complications of pancytopenia; specifically anaemia, haemorrhage and infection or from transformation to Acute Myeloid Leukaemia. More recently, it has been recognised that multiply-transfused patients with MDS develop iron-overload which may contribute considerably to morbidity and mortality, especially in lower risk subgroups who tend to have a longer life expectancy. Despite the recognition of iron overload due to transfusion in MDS patients, and evidence that ineffective haematopoiesis may result in defective utilization of iron, there is little data in the literature concerning iron accumulation already present at diagnosis. *Aims.* We sought to investigate ferritin levels in patients at presentation of MDS as a marker of iron overload. *Methods.* We performed a retrospective analysis of all patients presenting to our haematology service with a new diagnosis of MDS between 1996 and 2009. We recorded date of diagnosis, WHO 2008 Classification, ferritin value at diagnosis, transfusion status at diagnosis and cause of death for each patient. *Results.* Of 114 new diagnoses of MDS between 1996 and 2009, ferritin values were available for 82. The range of ferritin values at presentation was 3-5000ng/mL (normal range 15-200 ng/mL). Mean ferritin was 481ng/mL. Forty four patients (53%) had a ferritin level greater than the upper limit of normal (>200ng/mL). Of these 44 patients with elevated ferritin, only 6 had received prior transfusions (14%) with only 4 patients having received >5 units (9%). The cause of death for all patients who died was identified - only one heavily transfused patient died from cardiac failure due to iron overload. *Summary/Conclusions.* We speculate that high ferritin levels at diagnosis in MDS results partially from increased gastrointestinal iron absorption as a result of ineffective haematopoiesis. However, in the Irish population, it may also relate to the high prevalence of heterozygosity and homozygosity for the common haemochromatosis mutations. Excessive alcohol consumption may also contribute towards a higher baseline ferritin in this population. Cultural trends and genetic background may influence the risk of iron overload in different MDS patient populations. Iron chelation therapy (ICT) has been shown to reduce ferritin levels and may improve survival in patients with low- or intermediate-1 International Prognostic Scoring System scores. It has also been shown to improve cytopaenias. ICT has been recommended for low- and intermediate-1 risk patients who have received more than 20-30 packed red cell transfusions, for whom ongoing transfusions are anticipated and for those with serum Ferritin >2500 ng/mL. For Irish patients, and possibly for other patients of Celtic origin, where there is a high prevalence of haemochromatosis gene mutations, it may be useful to consider ICT earlier in the disease course.

1407

MYELODYSPLASTIC CHANGES AFTER ALEMTUZUMAB TREATMENT: MONOCLONAL ANTIBODY-INDUCED?

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Background. The myelodysplastic syndromes (MDS) comprise a heterogeneous group of malignant stem cell disorders characterized by dysplastic and ineffective blood cell production with a variable risk of transformation to acute leukemia. These disorders may occur *de novo* or arise between one and 5 years after exposure to potentially mutagenic therapy, named t-MDS (radiation exposure or after chemotherapy - alkylating agents or topoisomerase II inhibitors in most of the cases, but also purine analogs, like Fludarabine, a well known agent used in Chronic Lymphocytic Leukemia [CLL] treatment). Myelodysplastic changes after immunotherapy (i-MDS) are reported in the literature sporadically. Alemtuzumab (Campath-1H) is a therapeutic monoclonal antibody (Mab) that recognizes the CD52 antigen. CD52, a cell surface glycopeptide, is expressed on virtually all human lymphocytes. The highest expression is on CLL cells and the lowest on normal B cells. CD52 is expressed on all CLL cells. In CLL cells Alemtuzumab induces apoptosis. It is able to eradicate minimal residual disease (MRD) and achieve clinical response in high-risk relapsed patients. Adverse events related to alemtuzumab include acute first-dose reactions, infectious complications and rare hematologic toxicity. Prolonged lymphopenia is well known, but myeloid toxicities are less known. *Methods.* We treated five patients with relapsed CLL by Alemtuzumab (as second or third line of treatment). *Results.* In two patients there was a complete response, but myelodysplastic changes, which were not present before this treatment (when a massive infiltration by small lymphocytes occupied up to 90% of the bone marrow) were present post-treatment, especially in myeloid cells. These two patients developed serious infections and died in a short time. Myelodysplasia post Mab treatment is very rare. *Conclusion.* Myelodysplastic features post alemtuzumab treatment (Mab) are an even more rare event. The mechanism is unknown. It is not clear if this myelotoxicity is linked directly to Mab treatment or if it was present earlier, linked to Fludarabine or Cyclophosphamide, but expressed mainly in the progenitors cells, which were inhibited by the small lymphocytes and only after lymphocyte infiltration eradication expanded to all cells. Only three cases were described previously, not in CLL patients (in T-cell lymphoma). It is too early to establish a new medical term: i-MDS.

1408

LENALIDOMIDE IN A COHORT OF VERY ELDERLY PATIENTS WITH MYELODYSPLASIA AND DEL(5Q): A SINGLE CENTRE EXPERIENCE

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Background. Lenalidomide is a well-established treatment for myelodysplastic syndromes with del(5q) isolated or associated with other abnormal karyotypes. Little is known about the use of Lenalidomide in patients older than 75 years old (hereafter we referred to as very elderly patients). *Aims.* The aim of our retrospective analysis was to evaluate the efficacy and the feasibility of lenalidomide therapy even in very elderly patients, who often present comorbidities and low tolerance to anaemia, and for whom no alternative therapy is available. *Methods.* From November 2008 to date we treated four very elderly patients (median age: 81 years old, range 76-88) with a diagnosis of myelodysplasia and del(5q) and a low-int-1 IPSS risk score with Lenalidomide. Three patients received a diagnosis of "5q- syndrome", while another patient had a del(16)(q22) together with del(5q-). All the patients were transfusion-dependent at the time we started treatment (median transfusion requirement: 4 units/month, range 2-6). *Results.*In all four patients we observed a complete erythroid response according to IWG criteria (2006) and the median time to achieve the response was 7.5 weeks (range 4-10 weeks). Transfusion-independence had a variable duration (from 24 to over 65 weeks). All the patients achieved a partial cytogenetic response. In Patients 1 and 2 Lenalidomide treatment (10 mg/day for 21 days every 4 weeks) was well tolerated and is still ongoing. In Patient 3 treatment was stopped after 10 days due to the onset of serious agitation and panic attacks; regardless this patient had a transfusion-independence of 6 months. When anaemia worsened she started treatment again and had the same toxicity, so she stopped the drug after 15 days and had another 6 months of transfusion-independence. In Patient 4 Lenalidomide was stopped after 15 days despite a good tolerance due to a worsening of clinical conditions, but 4 weeks after treatment discontinuation she achieved a complete erythroid response. She remained in good clinical condition and transfusion-free for one year. *Toxicity.* as reported in the literature, we observed haematologic toxicity in 50% of patients, however in all cases there was a spontaneous recovery without severe infectious and hemorrhagic complications. Only in one patient the use of G-CSF was required. In one case treatment was stopped due to the onset of psychiatric complications. *Conclusions.* Lenalidomide therapy was effective and well-tolerated in this small cohort of very elderly patients. We observed a time of onset of the response longer than that reported in literature (7.5 weeks *vs.* 4.7 weeks) and a durable response in patients who continued the therapy without interruption (50% of cases); the other 2 patients had a delayed, yet durable erythroid response. Al the patients had a partial cytogenetic response. Lenalidomide therapy is feasible and effective

even in very elderly patients with myelodysplasia with del(5q) and/or other abnormal karyotypes.

1409

OXIDATIVE STRESS IN BLOOD PLASMA OF PATIENTS WITH MYELODYSPLASTIC SYNDROMES

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The myelodysplastic syndromes (MDS) constitute a heterogeneous group of clonal stem cell disorders characterized by ineffective hematopoesis, associated with cytopenias, leading to serious morbidity plus the additional risk of leukemic transformation. MDS are associated both with autoimmunity and increased oxidative stress. Inducible NO synthase is expressed in MDS patients. Autoimmune diseases with hyperhomocysteinemia may mediate oxidative cell damage by reactive oxygen species (ROS) production. We estimated concentrations of nitrites and nitrates and the presence of S-nitrosocompounds in plasma of MDS patients. To evaluate ROS production and its influence on plasma proteins we investigated their oxidative changes, plasma levels of homocysteine, other low-molecular weight thiols and malondialdehyde (MDA). Blood was collected into EDTA-coated tubes and plasma was obtained by centrifugation. Oxidized proteins in both MDS patients and control plasma were identified using biotin-avidin technology and mass spectrometry. Plasma levels of low molecular weight thiols or malondialdehyde were analyzed using HPLC of fluorescent derivatives. The concentrations of nitrites and S-nitrosothiols in plasma were estimated using HPLC of fluorescent 1-[H]-naphthotriazole produced by reaction with 2,3-diaminonaphtalene. S-nitrosoproteins in both MDS patients and control plasma were identified using biotin-switch method and mass spectrometry. We found increased concentration of homocysteine and MDA in patients plasma with MDS. Slightly increased concentrations of nitrites and S-nitrosothiols as compared with control samples were also found in plasma of patients with MDS. In our previous work we found changed platelet responses to various stimulants at the presence of either prooxidant or antioxidant species. It seems that the oxidation stress can contribute to the described defective platelet aggregation in MDS. *Acknowledgement.* This study was supported by Grants NS10633-3/2009 and MZ 02373601 from the Ministry of Health, Czech Republic, by Grant KAN200670701 from the Academy of Sciences, Czech Republic, and by Baxter, Czech Republic.

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IRON-CHELATION THERAPY IN MDS/IMF PATIENTS: DOES IT REALLY IMPACT ON TRANSFUSION REQUIREMENT?

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Background. Hematological improvements in MDS (myelodysplastic syndromes) or IMF (idiopathic myelofibrosis) transfusions dependent patients during iron-chelation therapy have been sporadically reported. However actual incidence, impact and potential predictive clinical factors of this phenomenon are still unknown. *Aims.* To extimate the actual impact of iron-chelation therapy on transfusion requirement or on other hematological parameters in MDS/IMF patients. *Methods.* A retrospective analysis was performed in MDS/IMF transfusion dependent patients to evaluate possible hematological improvement during iron-chelation. Transfusion requirement was defined as number of RBC units transfused every three months during the iron chelation period and compared with baseline requirement, for an overall period of one year. Platelets counts were also recorded every three months from beginning of iron chelation and compared to baseline ones. According to IWG criteria, erithroid response was defined "major"in case of transfusion independence; "minor"in case of 50% decrease in transfusion requirements. In thrombocytopenic patients (plt <100.000/mm^3) a "major"platelet response was defined as absolute increase of 30.000/mm^3 (for platelet transfusion-dependent patients, as stabilization of platelet counts and platelet transfusion independence); a "minor"platelet response was defined as 50% or more increase in platelet count with a net increase greater than 10.000/mm^3 but less than 30.000/mm^3. *Results.* From November 2007, eleven patients (10 low-risk MDS and 1 IMF) underwent to iron-chelation therapy in our institution. Only 8 of them had a follow-up sufficient for evaluation. They were treated with Deferasirox (7 cases; from 5 to 20 mg/kg/die) or Deferoxiamine (1 case; 30 mg/kg s.c. for 3-4 times/week). Average baseline ferritin level was 1837 ng/mL (SD 1433); after 9 months of chelation-therapy it was reduced to 1323 ng/mL (SD 897). No "major"erithroid responses were observed. "Minor"erithroid response (50% reduction of RBC units transfused) was reached after 6 months in one patient, after 9 months in two patients and after 12 months in two patients. Three patients had no improvement in transfusion burden. Only one patient was thrombocytopenic. He had a major platelet response after 3 months. Loss of erithroid response was observed in one patient after six months from time of response. The only platelet response was enduring at end of observation. All responders were affected by MDS and treated with Deferasirox. These preliminary results do not allow to identify clinical factors that can predict hematological improvement. *Conclusion.* Hematological improvement during iron chelation therapy was described, but currently considered as an infrequent phenomenon. In this small cohort, the impact of the erithroid response, even if "minor", seems to be relevant (about 60%) in MDS patients on Deferasirox treatment. Platelet response was also observed in a thrombocytopenic patient. Hematological improvements can occur as early as after 3 months of iron chelation. This interesting effect of iron chelation therapy in MDS/IMF deserves to be further investigated in a larger group of patients in order to explore possible predictive factors. In addition, a longer period of observation is required to evaluate the endurance of hematological benefits.

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RECOMBINANT HUMAN ERYTHROPOIETIN ALFA IN PATIENTS WITH LOW-RISK MYELODYSPLASTIC SYNDROMES, SELECTED ON THE BASIS OF A LOW PRETREATMENT SERUM ERYTHROPOIETIN CONCENTRATION

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Background. Epoetin alpha (EPO) continues to be the initial treatment of choice for most anemic patients with myelodysplastic syndromes (MDS). Subcutaneously, at a dosage of 80.000 U/week, has proven effective in relieving symptomatic anemia in 20-30% of patients (pts) with MDS. A low or intermediate-low risk score, according to International Prognostic Score System (IPSS) (Greenberg *et al.*, 1997) and low pre-treatment serum EPO levels are both associated with a higher probability of response. *Aims.* As rHuEPO is expensive, and the percentage of responder MDS pts is low, an attempt to select. MDS pts on the basis of basal serum EPO levels and IPSS score, is justified. *Methods.* From September 2005, 33 pts (27 males, median age: 66, range 48-84 yrs) with low- or intermediate risk MDS were treated with rHuEPO alpha for symptomatic anemia (Hb <10 g/dL). The pts received a high dose rHuEPO regimen: 40.000 U twice weekly, for the first month, followed by a single weekly dose of 40.000 as maintenance treatment, for at least 24 weeks. Only pts with a pre-treatment serum EPO <200 U/L were selected for rHuEPO treatment. *Results.* 27/33 pts (81.8%) showed a favourable response. 8 pts were transfusion-dependent. Among them, 6 showed a >50% decrease of their transfusion need. All the remaining 19 pts, with less severe anemia, not requiring transfusions, showed a clinical significant response (i.e. a >1g/dL increase of Hb). The median duration of response was of 13 (2-45) months, and 19 pts are still maintaining response under maintenance treatment. *Summary/Conclusions.* Although only 20-30% of MDS pts show a clinical significant response to rHuEPO, if pts are carefully selected on the basis of a low risk score (following IPSS) and a low (<200 U/L) pre treatment serum EPO level, the percentage of responses is higher (81.8%), this however does not exclude the use of EPO in other categories of MDS patients (Int-1 or EPO>200 U/L). Considering the high cost of EPO therapy, a selection of a cohort of low risk MDS patients with low serum EPO levels is necessary to maximize the cost-benefits ratio of therapy.

1412

PRECOCIOUS SECONDARY MYELODISPLASIA PRESENTING AS A CHRONIC MYELOMONOCYTIC LEUKEMIA IN A SEVERE CONGENITAL NEUTROPENIA YOUNG PATIENT

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Background. Severe congenital neutropenia (SCN) is a bone marrow failure with a clinical picture of early severe neutropenia and life-threatening bacterial infections. Maintenance treatment with recombinant

humanized G-CSF (rHuG-CSF) has changed the outcome and the quality of life of these patients. However a significant number of them will be affected by haematological cancer: acute myeloid leukemia (AML) and myelodisplasia (MDS). *Aim.* To present an interesting patient with severe congenital neutropenia and a novel ELA2 gene mutation and precocious development of secondary myelodisplasia. *Case.* A 6 month old boy was referred to our institution to study a severe neutropenia (0.3×10^9/L ANC) in the context of severe infection. His physical examination was normal with no growth impairment. The bone marrow aspirate showed maturation arrest at the promyelocyte/myelocyte stage, with no dysplastic signs neither other features. Cytogenetic analyses were consistently normal. Metabolic disorders, viral infections were ruled out. ELA 2 mutational status revealed a novel mutation (ELA2/ V36D). He started G-CSF (lenograstim) at 5mcg/Kg/day and was increased to 10 mcg/kg/day in order to maintain him around 1×10^9/L ANC. During follow up he presented several otitis and chickenpox. At 2 years old was admitted for a large splenomegaly (8 cm), anemia (64 g/L), thrombocytopenia (26×10^9/L) and monocytosis (5.6×10^9/L) and cutaneuos nodules in the scalp. The bone marrow showed moderate dysplastic changes in the erythroid and myeloid comparments and monocytosis. Cytogenetic analysis revealed monosomy 7 and KRAS mutation in the molecular analysis. *Discussion.* Since the introduction of rHu-G-CSF in SCN, AML and MDS have become the major events in these patients. Some works have correlated mutational ELA2 status with more severe phenotype and increased risk of hematological malignancies however further studies are needed. This patient shows the typical picture of secondary MDS presenting as chronic myelomonocytic leukemia. Bone marrow transplantation is advised in such patients. Open trials are evaluating the benefit of demethylating agents.

1413

RESPONSE TO 5-AZACITIDINE IN PATIENTS WITH MYELODYSPLASTIC SYNDROMES AND SECUNDARY AML: RESULTS FROM UNIQUE HOSPITAL

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Background. 5-azacitidine (AZA) significantly prolonged overall survival in higher-risk patients with myelodysplastic syndromes (MDS) in international phase III trial (AZA-001). However, data about efficacy of AZA in lower risk MDS are less consistent and only few studies have addressed this topic. Recently, FDA has approved AZA in all categories MDS, but EMEA has approved in high risk MDS. *Material and Methods.* we evaluate the efficacy and safety in all MDS groups and patients with secondary AML, non candidates to aggressive therapy. *Results.* In our institution, a total of 21 patients were treated with AZA since 2006. We evaluated 13 patients diagnosed according to WHO criteria as low/intermediate-1 International Prognostic Scoring System (IPSS) risk MDS, 4 patients as high/int-2 IPSS risk MDS and 4 secondary AML diagnosed patients. At baseline, the median age was 71.9 years (range 46-86), the male/female ratio 13/8. According to WHO classification, there were 1 RA, 6 RARS, 4 RAEB-1, 4 RAEB-2 and 1 MDS unclassified. Median time from diagnosis was 48 months (range 5-192). 85% patients were transfusion-dependent, 89% had received a prior treatment (rhu-EPO+G-CSF 55%, only rhu-EPO 34%). AZA was administered as single agent in 15 patients. Out of patients received a "standard"AZA dose of 75 mg/sqm/d subcutaneously during days 1-7, in a 28-day cycle. The median number of monthly cycles was 8.6 (range 2-26), and 61.9% completed at least 6 cycles. Grade 3-4 adverse events documented in these patients were myelosupression (20%), and injection site reaction (10%).

Hb pre-AZA	Hb max. post-AZA	Time to response months
6.9 (5.5-11.5)	10.7 (8.2-15)	3 (2-5)

Number Cycles Max. response	Number Cycles Lost response
5.3 (2-10)	11.8 (4-16)

Response	TI	PR	NR
	60%	30%	10%

TI: transfusion independence; PR: partial response; NR: non-response.

Response duration ranged from 1 to 8 months. There were no significant differences in response rate according to age, previous treatment, transfusion requirements, basal EPO and Hb pre-AZA. 2 patients were transformed to AML. 42.8% patients are death at moment of write this abstract. *Conclusions.* 1.- 90% patients achieved a hematologic response. 2.- Time to response is early (3months), although some patients response later (5 cycles or more). 3.- Efficacy and safety of AZA treatment is a valid alternative in low/int-1 risk MDS patients, although more studies are necessary. 4.- In secondary AML patients, AZA is a excellent alternative therapy in patients with co-morbidity and poor performance status.

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MYELODISPLASTIC SYNDROME WITH ADVERSE CYTOGENETIC ABNORMALITY IN CHILDHOOD: GOOD RESULTS OF ALLOGENIC HSCT

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Introduction. Myelodisplastic syndrome (MDS) is the most common hematopoietic malignancy in adults, but it is a very uncommon disorder in children and adolescents. The most frecquent MDS subtype in children is refractory cytopenia (RC). Allogeneic hematopoietic stem cell transplantation (alloHSCT) is the only potentially curative treatment, mainly when an adverse kariotype is present. However, scarce literature have been reported in alloHSCT and MDS with cytogenetic abnormalities. Monosomy 7 is the only chromosomal abnormality strongly suggestive of MDS in children. *Objectives.* We report our experience in all cases of MDS with adverse cytogenetic abnormality in children who were submitted to allogeneic HSCT in our hospital from 1999 to 2009. *Patients, materials and Methods.* A total of 4 MDS associated with an adverse cytogenetic abnormality have been diagnosed. All 4 patients were female, with a median age of 11.5 years old (10-14 years old). MDS were primary in 3 patients (1 refractory cytopenia with multilineage dysplasia [RCMD], 1 refractory anemia with excess of blasts type 2 [REAB-2] and 1 refractory anemia with excess of blasts type 1 [REAB-1]) and secondary in the remaining one (1 case of chemotherapy-related secondary REAB-1). Discovered karyotypes were t(X;20)(q13.1q13.3) [this karyotype had not ever been reported in children with MDS, as far as we know]; 47,XX(+8) y 45,XX(-7). The secondary case had got a complex karyotype with 2 cellular populations: 46, XX, del (5)(q14), -9, -18, -21q / 44, XX , del (5)(q14), -9, 17p-, -18, -21, 21q-, -22. The 2 fist cases received treatment with Cyclosporine A (CsA) and acute mieloblastic leukaemia (AML) intensive chemotherapy regimen before allogenic HSCT, without any response. The other two patients received allogenic HSCT as a first line of treatment. 2 patients received unrelated donor allogenic HSTC and 2 patients received HLA identical sibling related donor allogenic HSTC. In 2 cases, bone marrow was the source of HSC and peripheral blood was the source of HSC in the other 2 cases. Conditioning regimen was busulfan / cyclophosphamide in all cases. The Graft *vs.* host disease (GVHD) prophylaxis was made by CsA in 2 cases and by CsA and methotrexate in the other 2 patients. 3 patients developed acute GVHD (2 patients developed cutaneous acute GVHD and the other one developed cutaneous and intestinal acute GVHD). All of patients remain alive in a complete remission with a median follow up of 9.5 months (2-120 months) post-allogenic HSCT. Discussion: The karyotype is the main prognostic factor of MDS in childhood. Monosomy 7 and complex karyotype are strongly suggestive of adverse prognosis and high probability of progression to acute mieloblastic leukaemia. In contrast to monosomy 7, patients with trisomy 8 or normal karyotype may experience a long stable course of their disease. Allogenic HSTC is the only curative therapy and is the treatment of choice for patients with monosomy 7 or complex karyotypes early in the course of their disease. Immunosupresive therapy could be a successful initial strategy for children with other karyotipes. Responses are better in children with a normal karyotype. *Conclusions.* - MDS is a very uncommon diagnosis in childhood. - Cytogenetic analysis is essential in diagnosis and treatment of this type of patients. - Our report proves successful results of the allogenic HSCT in patients with adverse cytogenetic abnormalities. That should be indicated as first line therapy when a donor is available. - Immunosupresive therapy and myeloid acute leukaemia chemoteraphy are not effectives in our patients. Its benefit before alloHSCT must be clarified.

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MULTIDIMENSIONAL FLOW CYTOMETRY (MDF) TO IDENTIFY NORMAL AND ABNORMAL MYELOID, MONOCYTOID, ERITROID AND BLASTS POPULATIONS IN MYELOPROLIFERATIVE DISORDERS (MPDS) AND MYELODYSPLASTIC SYNDROMES (MDSS)

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Background. in our opinion MDF represents a highly reproducible and objective way of assessing the antigen expression in myeloid or monocytoid maturation as well as in benign reactive or malign proliferation or in normal marrow regeneration through analysis expression of multiple antigens on a single cell to identify normal or abnormal patterns of proliferation. The aim of this study is to correlate the normal antigen expression during haematopoietic development as determined by MDF with the dysregulation of haematopoiesis observed in MDS and MPDs. *Methods.* We have elaborated a multidimensional immunofluorescence protocol; forward scatter (FSC) and side scatter (SSC) were collected along with 7-8-color antibodies combination to generate 9-10 parameters per cellular event. multicolor flow cytometric immunophenotyping was performed on cytometer: Cyan ADPTM (Beckman Coulter). The following panel of antibodies was used in all of the cases: CD66b-Fitc/CD33-Pe/CD45-AmCyan/CD11b-Pe-Cy5/CD13-Pe-Cy7/CD10-APC/CD16-APC-Cy7/CD19PB; CD3-Fitc/CD16+CD56-Pe/CD45PerCp/CD4-Pe-Cy7/CD19-APC/CD8-APC-Cy7; CD64-Fitc/CD56-Pe/CD45-AmCyan/CD11b-Pe-Cy5 /HLADR-APC/CD14-APC-Cy7; TdT-Fitc/CD33-Pe/CD45-AmCyan/CD34-PerCpCD38-Pe-Cy7/Mpo-APC; CD36-Fitc/CD235-PE/CD45-AmCyan/CD71-APC. *Results.* We have been able to design combinations of antibodies to distinguish normal from abnormal maturation and the expression patterns of CD10, CD16, CD13, CD33, CD64, CD66b, and CD11b which we observe in MDSs are able to identify maturational anomalies and asincronous antigen expression. *Conclusion.* Our results indicate that multicolor flow cytometry allows to distinguish normal from abnormal myelopoiesis and also aberrant immunophenotype ($CD10^+$, $CD34^+$, $CD56^+$etc) is particularly useful in detecting and distinguishing leukemic from normal recovery blasts with high sensitivity and specificity respect to the patterns described in literature with three or four color flow cytometry.

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CLINICAL-BIOLOGICAL PROFILE OF A GROUP OF PATIENTS WITH PLASMACYTOMAS: A REVIEW OF HOMING BEHAVIOUR IN 68 PATIENTS WITH MULTIPLE MYELOMA

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Background. Extramedullary infiltration at diagnosis or throughout the disease course of patients with multiple myeloma affects the therapeutic strategy and often shadows prognosis. There are numerous studies linking homing behaviour of plasma cell to stage, antigen expression and treatments received, so far without any parameter that allows us to predict this behaviour. *Aims.* 1. To set extramedullary infiltration rate over the hospitalization events in patients with multiple myeloma at our center. 2. Analysis of type of myeloma, Salmon and Durie stage, inmunophenotype and treatments received in these patients. 3. To correlate these results with the group of patients without plasmacytomas. 4. To develop a possible profile of patients with myeloma which present or would be able to present extramedullary involvement in future. *Methods.* Sixty-eight patients with multiple myeloma diagnosed between 2006 and 2008 in our hospital were reviewed. We analyzed presentation of plasmacytomas at diagnosis or during clinical course, age, sex, type of myeloma, inmunophenotype (CD56, CD38++) and treatment on each group. We compared this group with the one without extramedullary infiltration using stadistic software SPSS (version 17.0). *Results.* Patients with extramedullary infiltration were 22 (32%) and at diagnosis 11 (16%) of total. In this group the proportion male to female was 12:10 and median age 59.2 years. The distribution of isotypes was IgG 13 (59%), 5 kappa and 8 lambda; IgA 6 (27%), 3 kappa and 3 lambda; Bence Jones 3 (13%), 1 kappa and 2 lambda. Salmon and Durie stages were IIA 8 (36%), IIA 6 (27%), IIB 2 (9%), IIIB 6 (27%). Inmunophenotypes: CD 56^+ 13 (68%) and CD56- 6 (32%). Four of them were $CD38^{++}$ (3 not analyzed). Treatments received were Thalidomide 11 (50%), Bortezomib 17 (77%), Lenalidomide 3 (13%), autolog stem cell transplantation 3 (13%). In the group without plasmacytomas (68%) proportion male/female was 23:23 and median age 56.8 years. IgG 31 (67.3%), 24 kappa and 7 lambda; IgA 10 (21.7%) 8 kappa and 2 lambda; Bence Jones 5 (10.8%) 3 kappa and 2 lambda. Stages: IA 2 (4.3%), IIA 21 (45.6%), IIIA 14 (30.4%), IB 1 (2.1%), IIB 1 (2.1%), IIIB 6 (13%). Analyzed inmunophenotypes (15 not available): 29 patients were $CD56^+$ (93%), 2 CD56- (6.4%) and 7 CD 38++ (22.5%). Treatments in this group were Thalidomide 22 (47%), Bortezomib 34 (73%), Lenalidomide 4 (85%) and autolog stem cell transplantation 23 (50%). There was significative difference in stadistic analysis between groups with extramedullary infiltration and without it in type of myeloma and CD56 expression (lambda/CD56: 59.1%/27.3% *vs.* 23.9%/4.3%, $P<0.05$). *Conclusions.* 1. Incidence of extramedullary infiltration observed in patients with multiple myeloma was high (one-third part). 2. There was no association between extramedullary infiltration in myeloma and sex, age or influence of treatment received, but this group presented more renal disease associated. 3. Patients with lambda myelomas and without CD 56 expression had more frequently plasmacytomas in our series and this could be related to homing behaviour. 4. Extramedullary infiltration carried a worse prognosis and lower possibilities of reaching an autolog stem cell transplantation.

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FUNCTIONAL ANALYSIS OF CYTOPLASMIC YB-1 IN MULTIPLE MYELOMA: TRANSLATIONAL CONTROL OF ANTI-APOPTOTIC PROTEINS

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The Y-box Binding Protein-1 (YB-1) is an oncogenic transcription/translation factor belonging to the evolutionarily highly conserved family of cold-shock domain proteins. YB-1 binds to DNA as well as RNA and fulfils pleiotropic cellular functions, including the regulation of proteins involved in cellular growth, survival and stress response. In transgenic mice, mammary gland specific over-expression of human YB-1 provokes breast cancer with a 100 % penetrance. YB-1 knockout experiments in mice showed that a homozygous deletion is lethal and a heterozygous YB-1 deletion is accompanied with an increased sensitivity to cisplatin and mitomycin C. YB-1 over-expression can be detected in many human malignancies including colorectal carcinoma, prostate cancer, osteosarcoma, breast cancer and multiple myeloma (MM). Compared to normal plasma cells YB-1 is strongly expressed in 30-50 % of primary MM samples. Over-expression of the protein was seen in a proliferative subset of primary MM cells, which are characterized by Ki67. Although eukaryotic Y-box binding proteins were originally identified as transcription factors binding to Y-box sequences in promoters of a variety of genes, the protein itself is predominantly localized in the cytoplasm of primary multiple myeloma cells and MM cell lines. To improve the understanding of YB-1 function in the cytoplasm we performed immunoprecipitation of YB-1 and subsequent analysis of the YB-1 bound mRNAs using gene expression arrays. Here we present the data of identified mRNAs bound to YB-1 in different MM cell lines and the adjacent functional analysis of promising protein candidates, like Mcl-1 and TCTP in multiple myeloma. This was accomplished by immunohistochemical investigation of primary samples, protein knockdown-/over-expression in MM cell lines, western blot analysis, the detection of mitochondrial membrane potential and caspase activation. The knockdown of YB-1 in MM cell lines led to a strong decrease of the protein level of the tested candidates while the mRNA level remained unaffected, validating their accuracy. The translational down regulation of these anti-apoptotic proteins caused the induction of apoptosis with subsequent activation of effector caspases, like caspase 3. In conclusion we state, that YB-1 can function as a RNA-chaperone in multiple myeloma stabilizing or enhancing the translation of anti-apoptotic proteins, thus assuring the pro-survival phenotype of MM cells.

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HETEROGENEOUS EFFECTS OF CXCR4 ANTAGONISTS ON THE PROLIFERATION OF MYELOMA CELLS

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Background. AMD3100, an antagonist for chemokine receptor CXCR4, induces the peripheral mobilization of haematopoietic stem cells. It also induces the segregation of myeloma cells in the bone marrow microenvironment, which should enhance the chemosensitivity of the cells. Based on these observations, AMD3100 is being considered for clinical

use. We have previously shown that AMD3100 induced the proliferation of myeloma cells in a short-term culture. *Aim.* We explored whether the proliferation-enhancing effect of AMD3100 is common to all the CXCR4 antagonists. *Methods.* Myeloma cell lines (RPMI8226 and U266) and CD138+ primary myeloma cells were incubated in serum-free medium with or without three CXCR4 antagonists (AMD3100, T140 and T22) at the concentrations of 0.1 μM to 10 μM for up to 14 days. Cell proliferation was measured by a modified MTT assay and cell apoptosis was analyzed by flow cytometry using annexin V. *Results.* All the CXCR4 antagonists markedly inhibited the stromal cell-derived factor-1 (SDF-1)-induced chemotaxis of myeloma cells. In addition to CXCX4, myeloma cells expressed CXCR7 to various degrees. All the antagonists induced internalization of CXCR4. However, AMD3100, but not T140 and T22, induced internalization of CXCR7 in these cells, indicating that only AMD3100 binds to CXCR7. SDF-1 or I-TAC, a ligand for CXCR7, on its own did not stimulate the proliferation of these myeloma cells, nor did it rescue the cells from apoptosis induced by serum deprivation. In 3-day cultures, AMD3100 (10uM) increased the number of cells up to 2-folds compared to controls, which was not abrogated by pretreating the cells with pertussis toxin. In contrast, T22 (1 μM-10 μM) decreased the number of cells by up to 35%, and abrogated the AMD3100-induced early proliferation enhancement. T140 (0.1 μM - 10 μM) did not affect the cell proliferation or apoptosis at all. In extended cultures of these cells for up to 14 days, AMD3100, but not T140 or T22, induced a marked decrease in the number of cells after 5-7 days. Taken together, AMD3100 exerts dual effects, initially enhancing and subsequently inhibiting the survival and proliferation of the cells. Adding SDF-1 at the beginning and middle of the incubation did not affect the early increase or later decrease in the number of cells induced by AMD3100, whereas it diminished the early inhibition of the cell proliferation induced by T22. T140 or T22 did not affect the cell proliferation after 5 to 7 days of cultures. The initial proliferation-enhancing effects of AMD3100 were not changed by knocking-down CXCR4 using the siRNA technique, whereas knocking-down CXCR7 significantly delayed the enhanced proliferation. In contrast, knocking-down CXCR4, but not CXCR7, partially restored the T22-induced decrease in cell proliferation. *Conclusions.* The effects of CXCR4 antagonists on the proliferation of myeloma cells are not uniform. Given that AMD3100 exerts dual effects, early enhancing and later inhibiting myeloma cell proliferation, whereas T22 exerts only early inhibition *in vitro*, combination of these two antagonists might be helpful in clinical use in myeloma patients.

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NONSECRETORY MYELOMA - SECRETORY POTENTIAL & CYTOGENETIC CHANGES

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Background. A rare variant of multiple myeloma is the nonsecretory form (NSMM), characterised by the absence of the detectable M-component in the serum and/or urine, with the related difficulties by diagnosing and the subsequent monitoring of the disease progress, which tends to be the reason why such patients are eliminated from clinical research studies. *Aims.* The study aimed at assessing the benefit of determining the serum levels of free light chains (FLC) in a group of NSMM patients, and evaluating the present cytogenetic changes. *Methods.* The examined group consisted of 8 patients who met the NSMM criteria of the International Myeloma Working Group. The serum levels of free light chains were determined by means of the FreeliteTM system. To determine the chromosomal changes, classic cytogenetic analysis and FICTION method were used with locus-specific probes 1q21/1p36 (Kreatech, MP Biomedicals, CA, USA), IgH, RB1, t(4;14), t(11;14) and centromeric probes for chromosomes 7, 9, 11, 15 and 17 (Abbott-Vysis, Downers Grove, IL, USA). *Results.* Pathological serum levels of free light chains κ or λ and their mutual ratio - κ/λ ratio were detected by 4 patients, namely in a quantity allowing for regular monitoring of the disease progress by means of the FreeliteTM system, whereas 2 patients revealed serum levels of FLC within the standard range, and a decrease in both FLC below the standard range was recorded in 2 cases. RB1 gene deletion was found by 6 patients together with translocation t(11;14), while this change was by all real, purely non-secreting forms. In addition, 2 of those patients revealed gain of 1q21 region. In one patient polyploidy and trisomy of chromosome 15 was detected. In one patient the cytogenetic analysis could not be made, due to multifocal character of the disease. *Conclusions.* Determining the serum levels of free light chains allows the additional detection of the secretion of light chains by part of the NSMM patients, leading to a reclassification of NSMM to an illness of κ or λ light chains. Consequently, this becomes an irreplaceable examination for monitoring the disease progress. Translocation t(11;14) and RB1 gene deletion seems to be the dominant cytogenetic changes by NSMM.

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CD40 LIGAND LEVELS IN THE SERUM OF PATIENTS WITH MULTIPLE MYELOMA CONSTITUTE A MARKER OF BONE MARROW PROLIFERATIVE ACTIVITY

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Background. The CD40 is a member of the TNF receptors superfamily expressed on antigen presenting cells such as B cells. Activation of CD40 by its ligand (CD40L) causes activation of B cells that leads to proliferation, switch and plasma cell differentiation. In malignant plasma cells, its activation is associated with increased aggression and proliferative capacity of the disease. CD40L is primarily expressed on activated CD4 (+) T cells. There is evidence that it promotes the adhesion and hence growth of myeloma cells. It has been shown that CD40L competitors have significant anti-myelomatic action. *Aim.* The purpose of this study was to measure soluble levels of CD40L in myeloma patients and to assess whether these could be an indicator of proliferative activity of multiple myeloma as expressed by the cell proliferation marker Ki67 and the secretion of growth factor hepatocyte growth factor (HGF). *Methods.* 43 patients with multiple myeloma and 15 healthy controls were studied. Patients were staged by Durie-Salmon system. Serum samples were collected from all patients at the diagnosis. We have also performed bone marrow biopsies prior to treatment, which assessed the expression of proliferation marker Ki-67 in plasma cells. Serum levels of CD40L and HGF were measured by ELISA. The percentage of Ki67 expression on plasma cells in bone marrow was measured by immunohistochemistry. For the statistical analysis of results we used the non parametric method (Mann Whitney and Wilcoxon) for comparison between patients and healthy controls and between different stages. The statistical correlation Spearman's rho was used to compare the identified factors. *Results.* CD40L and HGF serum levels as well the percentage of Ki67 positive plasma cells were significantly higher in the myeloma patients compared to controls (P<0.001). Serum HGF and CD40L levels and the percentage of Ki67 positive plasma cells were proportionally increased in accordance with the disease progression (CD40L: 505± 408pg/mL for stage I, 689±469pg/mL for stage II and 1471±651pg/mL for stage III and 242±99pg/mL for controls, P<0.001), (HGF: 821±372pg/mL for stage I, 1579±445pg/mL for stage II, 2608±1513 pg/mL for stage III and 471±128pg/mL for the controls, P<0.001). Serum CD40L levels in myeloma patients showed good correlation with HGF (r: 0.51, p 0.001) and Ki-67 expression in plasma cells. *Conclusions.* Our results demonstrate that CD40L plays an important role in the development of multiple myeloma as it is closely linked to the expression of the proliferation index of malignant plasma cells, Ki67 and the angiogenic index HGF. Given the contribution of HGF to promote angiogenesis in myeloma bone marrow, we support that the expression of CD40L could be an indirect marker of the angiogenic process. This is consistent with *in vitro* data in which activation of CD40 leads to secretion of angiogenic factor VEGF by malignant plasma cells.

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STROMAL MICROENVIRONMENT AND STEM CELLS NICHES IN MULTIPLE MYELOMA

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Background. Bone marrow stroma has a microenvironment which organized into bone and vascular niches of hematopoetic stem cells

(HSC). In multiple myeloma (MM), bone lesions and vascular rear-rangements determine HSC niche involvement into MM pathogenesis. *Aims.* The study described here examined the microenvironment of intramedullar stromal bone marrow in patients with MM. The emphasis was on the osteogenic cells role in the endosteal zone of trabecular bones and sinusoidal vessels. *Methods.* Stromal and hematopoetic tissue was sampled from the ilium for 79 patients with MM. The samples were examined for morphology/morphometric parameters and by electron microscopy. Proliferative activity of mesenchymal stem cells was evaluated using our proprietary *in vitro* cultivation method. Angiogenesis activity was evaluated using the microvessel density index (MDI). All patients were treated with bortezomib-containing combinations (BCC). *Results.* During the MM active phase, there was a reduction in adipocytes; reticular cell number in-creased; phagocyte activityof reticular cells increased; and local reticular sclerosis could be seen in the bone marrow. Centers of bone trabecular resorption were identified and the structure of endosteal colla-gen was disrupted in these centers. The banding of transverse collagen fibers was difficult to detect and some fiber banding disappeared completely and was replaced by extracellular matrix clumps. Both the number and the structure of endosteal osteogenic cells changed. The number of spindle-shaped osteob-lasts increased ($P<0.05$). Increased functional cell activity was confirmed by the appearance of cytop-lasmic organelles and the state of the nuclear chromatin. Increased proliferative activity of endosteal cells was also detected. The number of bone trabeculae was slightly reduced, but this reduction was not sta-tistically significant ($P>0.005$). The number of sinusoidal vessels was increased ($P<0.05$). MDI was also significantly increased. In patients treated with 6-8 cycles of BCC and achieved CR, bone marrow stroma samples showed decreased number of endosteum osteogenic cells and decreased destruction of collagen fibers. The MDI was significantly decreased. The sinusoid volume index did not differ from the index in healthy individuals. *Conclusion.* The results demonstrated significant changes of intramedullary stroma status and HSC nich-es in MM. The endostal osteogenic cells have two main functions: to maintain the HSC and bones. This functional ability is changed during MM. Decrease in bone tissue volume is related to both increased os-teoclast activity and abnormal bone formation function of endosteal cells. It is also possible that the he-mopoesis-inducing activity of endosteal cells is enhanced, which would support extension of the myeloma cell clones. Intensive angiogenesis and sinusoid changes were evidence of functional rearrangements in the vascular niche. Bortezomib regulates protein degradation, directly influencing stromal microenvironment cells, myeloma cell clones, and osteoclast activity. Endosteal zone status and blood flow were improved in patients with MM treated with bortezomib and achieved CR. This confirms the importance of bone and vascular niches in MM pathogenesis. Therefore, new drugs should target not just the myeloma cells themselves but also the activity of the bone and vascular niches. The status of the HSC niches is an important prognostic criterion for transplantation outcome.

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ROLE OF SEROTONIN REGULATION IN OSTEOLYTIC LESIONS OF PATIENTS WITH MULTIPLE MYELOMA

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Background. The biologic mechanisms involved in the pathogenesis of multiple myeloma (MM)-induced osteolytic bone disease are poorly understood. Physiological interactions between the serotoninergic and skeletal systems are implicated by clinical observations.[1-3] MM patients with evidence of osteolytic lesions exhibited an increase in the concentration of serum tryptophan and serotonin,[3] while that of tyrosine, dopamine, and noradrenaline was decreased.[3] We hypothesize a direct role of serotonin signaling in myeloma bone disease. The monoamine serotonin [5-hydroxytryptamine (5- HT)] has previously been investigated as a neurotransmitter, synthesized by a two-step pathway in which tryptophan hydroxylase is the rate-limiting enzyme. The brainstem-derived serotonin (BDS) positively regulates bone mass following binding to 5-HT2C receptors on ventromedial hypothalamic neurons. This is opposed by platelet-derived serotonin (PDS) which induces bone lysis and osteoclast activation. Circulating 5-HT is principally stored in platelet-dense granules. Immunoglobulins have been shown to induce platelet release a) when participating in immune reactions as antigen-antibody complexes or b) by nonimmune mechanisms such as coating of glass or polymethylmethacrylate beads. Aggregated immunoglobulins derived from all the IgG subclasses, isolated from healthy controls or myeloma patients, induce platelet granules to release serotonin (in the absence of antigen or particulate matter) in a dose dependent manner.[2] *Aims.* Increased circulating-serotonin levels released from platelets by immunoglobulin complexes may alter the RANK/RANKL ratio in the BM environment and promote MM osteolytic lesion. *Methods.* We explored the role of serotonin in MM bone disease by examining the levels of serotonin and a range of 20 cell signaling proteins in bone marrow core biopsies. We also measured circulating serotonin levels by HPLC in a pilot set of MM patients (n=5). We retrospectively examined 15 bone marrow core biopsies from patients with different clinical stages, using reverse-phase protein microarray (RPMA). RPMA is a reproducible, high-throughput system for protein signal pathway profiling, providing direct information about receptor phosphorylation and consequent activated network . *Results.* We evaluated the association of serotonin and adrenergic receptor levels with DKK1 and RANK which participate in bone loss, as well as with a variety of hormone and cytokine receptors, and a series of phosphorylated signaling proteins. 4 out of 5 samples in patients with osteolytic lesions had detectable serotonin. Positive correlations were noted between DKK1, Ezrin Thr567, Deptor, and serotonin in patients with osteolytic disease. *Summary.* These data suggest that the 5-HT system plays an important role in bone homeostasis through effects on osteoclast differentiation and can support the hypothesis that the serotonin system is involved in the pathogenesis of MM-induced bone disease. This insight could provide strategies for reducing osteolysis using agents which regulate circulating serotonin.

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PROGNOSTIC SIGNIFICANCE OF MORPHOLOGICAL EVALUATION OF TUMOR CELLS IN MULTIPLE MYELOMA

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Background. Multiple myeloma (MM) is the second most common hematological malignancy. It is caused by clonal proliferation of terminally differentiated cells of B-lineage. Morphology assessment including the determination of plasma cell percentage in the bone marrow remains one of the basic diagnostic procedures even in the era of genomics. The objective of this study was to evaluate the prognostic impact of the presence of different plasma cell morphological subtypes on overall treatment response and long-term survival. We also analyzed whether this parameter can be correlated to other conventional prognostic/predictive markers. *Patients and Methods.* Our cohort consisted of 139 newly diagnosed MM patients (pts) who subsequently underwent autologous transplantation (AT) within the 4W and CMG 2002 clinical trials in a single center. Percentage of plasma cell subtypes in the bone marrow was evaluated based on the progressive nucleolus analysis, assessment of nuclear chromatin, and the nucleus/cytoplasm (N/C) ratio. A combination of these elements permits differentiation of eight subtypes P000-P111 and four subclassifications. *Results.* Mature plasma cells (P000, P001) were found in 42.4% of patients; type I proplasmocytes (P010,P011,P100) in 38.1% of patients; and type II proplasmocytes (P101,P110) in 19.4% of patients. For patient undergoing AT, group of pts with overall therapeutic response ORR has lower number of mature plasma cell P000 group than pts without treatment response (median 24.0% *vs.* 36.0%; P=0,032). Patients with bone marrow infiltration by mature plasmocytes (P000 subtype) <10% had shorter overall survival compared to pts with P000 ≥37% (median 46.8 months *vs.* 77.8 months; P=0.020). The presence of proplasmocytes (P110 subtype) <3% was associated with longer time to progression compared to P110 ≥31% (median 54.6 months *vs.* 22.4 months; P=0.045). Patients with ISS stage 1 or 2 had lower percentage of P010 (type I) proplasmocytes than stage 3 patients (11.5% *vs.* 23.0%; P=0.030). In contrast, higher infiltration with P100 (type I) proplasmocytes and P101 (type II) proplasmocytes

was observed in ISS stage 1-2 patients compared with stage 3 patients (12.0% *vs.* 6.5%; P=0,015 for P100 a 1.0% *vs.* 0,0%;P=0,046 for P101). Patients without 13q14 deletion have had higher bone marrow percentage of mature P000 plasmocytes than patients with del 13q14 (35% *vs.* 13%; P=0.014). Del13q14 was also associated with lower counts of type II P110 proplasmocytes (36,5% *vs.* 6,0%; P=0,012). There was found no other association with evaluated chromosomal abnormalities including 1q21 gain, del(13)(q14), del(17)(p13), t(4;14)(p16.3;q32), and t(11;14)(q13;q32). *Conclusion.* Despite advances in high-tech genomic technologies, evaluation of plasmocyte infiltration of the bone marrow still belongs to basic diagnostic procedures in MM and further morphological subtyping of plasmocytes provides important prognostic information in MM patients treated with autologous stem cell transplantation.

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SERUM PROINFLAMMATORY CYTOKINES PROFILE IN MULTIPLE MYELOMA RELATED TO PATHOGENESIS OF BONE DISEASE

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Background. Bone disease is one of the most frequent complications in Multiple Myeloma (MM), and as consequence produce irreversible and invaliding lesions. The pathogenesis of bone disease is not completely known. There are several publications about the influence of different cytokines in the bone remodelling, by break the balance between osteoclastic/osteoblastic activities. Aims of the study: to analyze a cytokine panel in a group of consecutive patients diagnosed as MM according the International Myeloma Working Group, 2003 criteria and to compare the results between patients with an without overt bone disease and to study the profile change related to therapy response. *Patients and Methods.* Between June 2008-June 2009, a total of 40 consecutively MM patients were studied (46.0% females) in Haematology Department of Miguel Servet University Hospital; mean age: 70.2 (range: 66.3-74.0). According subtypes distribution: Light Chain: 6 (15.0%) IgG: 21 (52.5%); IgA 11(27.5%), double MC 2 (5.0%); the ISS (1: 9, 22.5%, 2: 21 52.5%, 3: 10 25.0%). Four different degrees of bone disease was established: 17 normal (48.58%); 6 osteopenia (17.14%); 1 osteoporosis (2.86%); 5 Lytic lesions (14.28%); 6 fractures (17.14%). The cytokine assay was performed in serum samples stored at -80ºC at diagnosis and the end of therapy, using Millipore human cytokines Kit according the established protocol by Luminex®100 platform. The profile cytokine panel used were: IL-4, IL6, IL-7, IL-10, IL-13, MIP-1α,MIP-1β, TNFα and RANKL. Previously we had compared the cytokine profile with a group of healthy population (n=36) and MGUS (n=36) group matched by age and gender. The results of the cytokine panel were compared between group's control/MGUS/MM, bone disease, immunochemical subtypes and ISS stage, haemoglobine, β2 microglobuline concentration, free light chain ratio in the MM group. Descriptive analysis and non-parametric test was performed using SPSS 15.0 and STATA SE v.10. *Results.* The cytokine profile of control/MGUS/MM was significant different for IL-4 (P=0.02), IL-13 (P=0.001), MIP-1α(P=0.03), MIP-1β(P=0.001) and TNFα(P=0.003). Considering only MM group the comparative analysis of profiles according ISS classification showed significant differences for MIP-1α(P=0.04), IL-13(P=0.02) and β2microglobuline *vs.* TNFα (P=0.002). No significant differences were observed for immunochemicals subtypes, free κ/λ ratio and haemoglobine. *Conclusions.* In our experience Luminex®100 technology is a sensible and accurate technique useful to determine cytokine profile in serum. A different significant cytokine profile was observed between control/MGUS MM and in MM group: β2microglobuline and ISS. It is necessary to perform more studies with more patients in order to confirm these results.

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AN INTERIM STUDY REPORT OF CD44 STANDARD MOLECULE AND ITS VARIANT ISOFORM EXPRESSION IN MGUS AND MM

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Background. Multiple myeloma (MM) is a neoplastic plasma cell (PC) disorder in which the malignant PCs accumulate in the bone marrow (BM); this has been defined by regulation of various adhesion molecules. Expression of adhesion molecules such as CD56 and CD138 prominently represented as a tool for PC identification and characterization in PC disorders. CD44 or CD44 standard molecule (CD44s) is restricted on haematopoietic cells and involved in various adhesive functions such as cell-cell interaction, binding to matrix and homing to inflammatory sites. Few studies documented the CD44s and CD44 variant (CD44v) isoforms expression in MM. We made an attempt to renew the interest of CD44s, CD44v4 and CD44v6 expression in MM. *Aims.* To evaluate the differential expression of CD44s, CD44v4 and CD44v6 in BM $CD19^+$ cells and BMPCs of MGUS and MM patients (pts). To analyze the clinical features that influence expression of CD44s and CD44v isoform in MM. *Methods.* In this pilot study a total of 43 pts were enrolled and diagnosed as 9/43 with MGUS, 14/43 with symptomatic MM and 20/43 with relapse. Five-color flow cytometric immunophenotyping was applied to screen CD44s, CD44v4 and CD44v6 expression in BMPCs and PC excluded $CD19^+$ cells. Following 5 color combination of MoAbs were used for phenotyping: CD44s/CD44v4/$CD44v6^-$FITC/$CD19^-$PE/$CD138^-$PercpCy5.5/CD56-PE-Cy7/CD38-APC. Estimation of statistical significance (P<0.05) between groups were performed using Mann-Whitney U test and correlation analysis was done by Spearman Rank Order correlations. *Results.* PCs were identified on the basis of $CD138^{++}CD38^+$ expression and PC excluded CD19+ cells were characterized as $CD138^-$ $CD38^-CD19^+$. Expression of CD44s and CD44v molecules in PCs and $CD19^+$ cells has been summarized in Table 1. In MGUS, MM and relapsed cases overall differential expression of CD44s, CD44v4 and CD44v6 in PCs and $CD19^+$ cells were observed; however it did not reach statistical significant difference. In accordance with several reports, statistically significant difference in the BMPCs of MGUS *vs.* MM (P=0.009) and MGUS *vs.* relapsed cases (P=0.017) were found. Notably for CD44s molecule, BM $CD19^+$ cells had higher frequency of CD44s expression compared to BMPCs (P<0.001). In univariate analysis, ≥ 3.5 mg/L of β2M (P=0.040) was found to be the influential factor for BM $CD19^+CD44s^+$ cells. Features such as <10% of BMPCs (P<0.01) and <30g/L of M-protein (P<0.01) were chosen as most influential factor for $CD44v4^+$ expression in BMPCs. More interestingly, inverse correlation had been observed between $CD44v4^+$ *vs.* BMPCs (R= -0.44; P=0.003) and $CD44v6^+$ *vs.* BMPCs (R= -0.34; P=0.021). *Summary.* This interim report revealed that there is no significant influence of CD44s, CD44v4 and CD44v6 expression with regard to distinguishing MGUS and MM. The unique finding from this study is inverse correlation between CD44v isoforms and BMPCs. We are expecting CD44v4 and CD44v6 isoform could serve as a prognostic marker in MM and moreover this should be confirmed in large cohort of pts.

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Table 1. Expression of CD44s, CD44v4 and v6 in MGUS and MM.

Group	BM $CD19^+$cells/Leukocytes %Median (range)	$CD19^+CD44s^+$ %Median (range)	$CD19^+CD44V4^+$ %Median (range)	$CD19^+CD44V6^+$ %Median(range)
MGUS	1.62 (0.7-4.61)	98.7(92.02-99.8)	2.61(0.95-4.72)	6.75 (1.55-15.16)
MM	1.08 (0.48-4.07)	98.5(90.5-99.6)	3.45(0.5-10.48)	8.19 (0.68-44.57)
Relapsed MM	2.28(0.15-10.13)	97.8(88.2-100)	5.31(1.03-18.54)	10.45(3.6-24.5)

Group	BMPCs/Leukocytes %Median (range)	PCs $CD44s^+$ %Median (range)	PCs $CD44V4^+$ %Median (range)	PCs $CD44V6^+$ %Median(range)
MGUS	0.49 (0.03-1.9)	96.38(81.7-98.8)	5.58(1.33-11.76)	13.18(2.13-22.25)
MM	3.6 (0.02-26.43)	92.7(17.5-99.3)	6.03(0.2-29.45)	9.4(1.64-53.57)
Relapsed MM	2.78(0.02-38.42)	93.15(5.3-99.9)	5.12(0.04-29.47)	9.52(0.46-41.05)

*Statistical significant difference was observed only in BMPCs; MGUS vs. MM (P=0.009) and MGUS vs. Relapsed MM (P=0.017).

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MATRIX METALLOPROTEINASES -2 (MMP-2) AND -9 (MMP-2), TISSUE INHIBITOR OF METALLOPROTEINASE - 1 (TIMP-1) AND-2 (TIMP-2) PRODUCTION AND EMMPRIN (CD147) EXPRESSION ARE INCREASED IN PATIENTS WITH MYELOMA MULTIPLEX AND DISPLAY CORRELATION WITH ADVANCED STAGE

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Introduction. Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases characterized by the activity to degrade of the extracellular matrix. Gelatinases : MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are members of MMP family involved in tumor initiation growth and metastasis. The activity of MMPs is controlled by their interaction with tissue inhibitors of metalloproteinases (TIMPs). Myeloma multiplex (MM) is a B-cell malignancy with the excessive bone resorption and the tumor invasion process inside and outside the bone

marrow. There is a little data about concentration of MMP-2 and MMP-9, their tissue inhibitors (TIMP-1, TIMP-2) and an activator emmprin in myeloma patients. *Aim.* The aim of our study was to estimate the bone marrow concentration of MMP-2, MMP-9, TIMP-1, TIMP-2 and emmprin expression in MM patients and whether these parameters are connected with the disease`s advanced stage. *Patient and Methods.* Forty -four patients: 27 men and 17 women, aged 48- 81 mean: 52.4 years with newly diagnosed MM were enrolled in the study. According to Durie and Salmon staging system 4 patients were in I stage, 10 in II stage and 30 in III stage. The control group enclosed 17 healthy subjects matched sex and age. Bone marrow samples were obtained at presentation. The bone marrow stromal cells (BMSCs), CD166 positive, were incubated in Mesencult. Supernatans were collected and stored at -80°C until measurement. Concentration of MMP-2 and TIMP-2 in supernatants was assessed by ELISA method. MMP-9 and TIMP-1 were assessed in bone marrow before culture by ELISA method. Expression of CD147 on CD166 positive cells was assessed by flow cytometry. *Results.* Mean concentrations of MMP-9, MMP-2 , TIMP-1 and TIPM-2 were significantly higher in MM patients than in normals. The MMP-9/TIMP-1 and MMP-2/TIMP-2 ratios were significantly higher in MM group than in the control. Moreover we revealed the positive correlation between concentration of MMP-2 and MMP-9 with advanced stage of MM according to Durie-Salmon staging system and number of bone osteolytic lesions. Expression of CD147 on BMSCs obtained from MM patients were significantly higher than on BMSCs in normal group. *Conclusion.* Our study revealed increased concentration of MMP-2, MMP-9, TIMP-1 and TIMP-2 in bone marrow of patients with MM and positive correlation with the disease advanced stage and a number of osteolytic lesions.

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STEM CELL MARKER NESTIN IS EXPRESSED IN PLASMA CELLS OF PATIENTS WITH MONOCLONAL GAMMOPATHIES

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Backround. Nestin, an intermediate filament protein is known to be a stem cell marker expressed in various embryonic and fetal tissues. Nestin expression is downregulated in adult terminally differentiated tissues. Currently, nestin expression has been detected in many tumors including multiple myeloma (MM). Nestin is supposed to be a cancer stem cell marker; its overexpression is related to malignancy and plays a crucial role in aggressive behaviour, migration and proliferation of several solid tumors. *Aims.* The aim of this study was to confirm nestin expression in plasma cells (PCs) of patients with monoclonal gammopathies (GMs). *Methods.* A total number of 37 MG patients (23M/14F; median age 68 years) represented by 92% (34/37) of MM patients, 5% (2/37) of MGUS patients and 3% (1/37) of non-secretory MM patients were included in this pilot study. Five patients without monoclonal gammopathy served as a control group. Bone marrow mononuclear cells were analysed by 3-color flow cytometry, and PCs were identified as $CD38^+CD138^+$ followed by direct staining for intracellular nestin. Nestin expression was assessed based on percentage of PCs showing positivity for nestin (Nes+PC) and median fluorescence intensity of nestin expression in PCs. Nestin expression was verified in 8% of MG patients (3/37) by immunocytochemistry and in 24% of samples (9/37) by western blots. *Results.* The whole group of MG patients had the median percentage of Nes+PC 31.3% (range, 0%-95.4%). In the control group, the median percentage of nes+PC did not exceed 1.5% (range, 0.3-2.7%). The median fluorescence intensity of nestin expression in PC was 4.2×10^2 (range, 0-6.8×10^3). Immunocytochemistry demonstrated nestin immunoreactivity in all analysed samples (3/3) as the network of nestin filaments or the diffused signal in the cytoplasm of PC. Western blot analyses confirmed nestin expression in 78% (7/9) of MM cases. *Conclusion.* Our results clearly confirmed nestin expression in PCs of MG patients. The percentage of nestin-positive PC and the median fluorescence intensity of expression ranged between patients. Prognostic/predictive significance of these differences requires an evaluation on a larger number of patients and correlation to clinical data. Based on current reports, we hypothesise that nestin could be a prospective marker of myeloma stem cells and a possible target of anticancer therapy. Further studies are required to confirm our hypothesis.
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A PRELIMINARY STUDY REPORT OF T REGULATORY CELLS IN PRE AND POST TREATED MYELOMA PATIENTS

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Background. T regulatory (Treg) cells play an important role in modulation and maintenance of overall immune response against infectious disorders and tumors. In various cancers including solid and hematological malignancies clinical anti tumour response has been degenerated due to expansion of Treg cells. Immune dysfunctions were also described well in multiple myeloma (MM) and now it is obvious to exploit the Treg cells in MM. *Aims.* The proposed concept of this study is to compare the frequency of $CD4^+$ Treg cells in newly diagnosed and treated/transplanted MM patients (pts). Evaluation of possible association of Treg cells to clinical features of MM. *Methods.* A total of 51 newly diagnosed MM pts were enrolled in this study after signing informed consent. Patients were clinically represented with different stages of MM (International Staging System: ISS 1=17%, ISS 2=39% and ISS 3=44%). A uniform treatment including thalidomide in combination with cyclophosphomide and dexamethasone (CTD) was given in four and eight cycles, respectively for younger (<65 years= 41%) and elderly (≥65 years= 59%) pts. Followed by 4 cycles of CTD regimen, autologus stem cell transplantation (ASCT) was offered to younger pts. $CD4^+$ Treg cells had been quantified based on four-color flow cytometric phenotyping for antigens CD4, CD25, CD127 and FoxP3. All pts peripheral blood (PB) and bone marrow (BM) samples were analyzed for $CD4^+$ Treg cells during diagnosis, as well for 17 treated and 8 transplanted pts. As a control group 20 healthy individuals PB $CD4^+$ Treg cells were also analyzed. *Results.* Immunophenotypisation revealed $CD4^+$ Treg cells as $CD4^+$ $CD25hi^+$ CD127dim/- $FoxP3^+$. Enumeration of Treg cells were performed on the basis of $CD4^+$ $CD25hi^+$ $FoxP3^+$ phenotype. Our results shown a paradigm of statistically significant increased frequency of PB Treg cells in MM pts compared to control group (median=5.48% *vs.* 3.82%; P<0.0001). Quantified PB and BM Treg cells of MM pts had shown significant positive correlation (R=0.70; P<0.0001). A pattern of reduction in the PB Treg cell frequencies were observed after eight cycles of CTD regimen, compared to pre-treatment (median=4.61% *vs.* 5.71%; P>0.05 [not significant]). Proportionately, PB Treg cell frequencies in transplanted pts were also found to be reduced than prior to transplantation (median=3.4% *vs.* 5.94%; P>0.05). Whereas, BM showed increased number of Treg cells in transplanted pts than at the time of diagnosis (median= 5.59% *vs.* 4.35%; P>0.05). In univariate analysis clinical parameters such as ≥ 30 g/L of M-protein and ≥ 3.5 mg/L of β2- microglobulin were chosen for increased frequency of PB and BM Treg cells, although it did not reach the statistical significance. *Summary.* As anticipated, CD4+ Treg cells were found to be increased more prominently in MM pts. From this preliminary study report no strong conclusion can be drawn but a paradigm of reduction in Treg cells was documented in post treated and transplanted pts. Therefore, the impact of Treg cells in pre and post treated MM pts must be acknowledged in large series of pts.
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CD45 EXPRESSION BY BONE MARROW PLASMA CELLS AT DIAGNOSIS IN MULTIPLE MYELOMA: CORRELATION WITH MINIMAL RESIDUAL DISEASE

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Background. Multiple myeloma (MM) is characterized by accumulation of clonal plasma cells (PCs) in bone marrow. CD45, a key regulator of antigen-mediated signalling and activation in lymphocytes, is present in early stages of PCs development. CD45 is now established as a critical component of the signal transduction machinery of lymphocytes. *Aims.* In the present work we are interested in tracking the pattern of expression of CD 45 by malignant plasma cells at diagnosis in comparison with the degree of minimal residual disease after induction by Thalidomide and Dexamethasone. *Methods.* CD45 expression on multiple myeloma plasma cells and minimal residual disease quantification were studied by flow cytometry (FCM). A total of 14 patients (13 H, 1F) were studied at diagnosis and after first line therapy. Inten-

sity of expression and the percentage of plasma cells are taken into account when determining the expression profile of CD45. Samples were considered $CD45^+$, if they present more than 20% of plasma cells with bright CD45 expression (>102). Response to treatment was evaluated by quantification of CD38 high $CD138^+$ $CD56^+$ cells. A lower threshold of 10-2 was considered as a good response. *Results.* At diagnosis, the average of plasma cell bone marrow infiltration was 35% by cytology and 25% by FCM. There is a correlation between the percentage of plasma cells estimated by cytology and FCM with a significant value (P=0.005). The expression of CD 45 was predominantly negative in 9 (64%) of 14 samples. Minimal residual disease separate two groups of patients: The first group: 6 patients with MRD>10^{-2} and a profile CD 45 predominantly negative (only one sample was CD45 positive) and a second group: 8 patients with MRD<10^{-2} with five samples CD45 positive. Negative expression of CD45 is correlated with bad response to treatment (P=0.05). *Conclusion.* In this study, we report a positive correlation between the expression of CD 45 and the therapeutic response. Further prospective studies are necessary to confirm this concept.

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DOES CD117 HAVE AN INDEPENDENT PROGNOSTIC VALUE IN PATIENTS WITH MULTIPLE MYELOMA?

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Background. Immunophenotyping by flowcytometry is known as a useful method in diagnosis and prognosis of multiple myeloma. Guidelines of European Myeloma Network (EMN) recommend a few markers to be assessed for prognosis, such as CD56, CD117, CD20, CD27, CD28. *Aim.* to assess prognosis value of CD117 and association with other biological features in patients with multiple myeloma. *Methods.* We have studied 22 consecutive, new diagnosed myeloma patients. Immunophenotyping analysis was performed on 4 colors BD FACS Calibur flowcytometer. For statistical analysis we used SPSS software. Mandatory panel recommended by EMN was used for diagnosis by flowcytometry, gating the CD38 and CD138 positive plasma cells; prognosis markers were considered CD20, CD56, CD117, CD45. *Results.* Sex ratio: 7 females, 15 males; average age=60 years. Durie-Salmon staging was as follows: stage II(60%); stage I(20%) stage III(19%), 1% smoldering myeloma. Regarding CD117 expression, we divided the patients in 2 groups: 11/22 patients (3 females, 8 males; average age=70 years) had poor prognostic immunophenotypic profile: CD117 positive, CD56 positive in 9/11cases; the other half - 11/22 patients (4 female, 7 male; average age - 57 years) had favorable prognosis profile: CD117 negative, $CD56^+$. Percentage of myeloma plasma cells existent in bone marrow is not different between the two groups of patients (ranging between 20-80%). We found a possible correlation of low expression of CD45 with low expression of CD56 on myeloma cells, and an unusual high expression of CD45 and $CD56^+$ on malignant myeloma cells and with good clinical outcome, also(P=0.05). In both groups, the most restriction was with kappa light chains (CD117 positive: kappa/lambda ratio - 10/1; CD117 negative: kappa/lambda ratio - 9/2). Immunoglobulin G was most frequently encountered in both groups (CD117 positive patients: 7 patients with IgG, 2 patients with IgM and 2 patients with IgA; CD117 negative group - 6 patients with IgG, 3 patients with IgE). Considering evolution, patients with positive expression of CD117 had mostly unfavorable outcome (7/11); from CD117 negative patients, 6/11 went worse. *Conclusions.* Our results on preliminary data suggest that CD117 has not a particular prognosis value as independent parameter in multiple myeloma.

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TUMOR NECROSIS FACTOR-ALPHA AND OTHER PROINFLAMMATORY CYTOKINES IN MULTIPLE MYELOMA

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Background. Excessive or inadequate expression of proinflammatory cytokines during the immune response may produce autoimmune phenomena at the inflammatory site by tissue destruction, consequently maintained by complex mechanisms in with the proinflammatory cytokines play a critical role. Multiple myeloma represents a disorders characterized by the presence of general inflammatory processes. Studies on peripheral bllod proved the presence of numerous proinflammatory cytokines (TNF-α, IL-1 and IL-6). *Aims.* We tried to assess the serum concentration of the proinflammatory cytokines: TNF-α, IL-1 and IL-6 as a mirror of type 1 response. *Methods.* We studied 28 multiple myeloma patients in a progressive stage of the disease, without any other autoimmune disease, other tumor or sistemic infection compared with a group of 28 healthy individuals, with a median age of 30 years. We performed immunoenzimatic determinations by ELISA technique, following the working protocol Quantikine, R and D System, Minneapolis, USA for the TNF-α and Endogen, Woburg, MA, USA for the IL-1 and IL-6 . We have determined the serum level of some proinflammatory cytokines (IL-1 α, IL-6 and TNF-α) in multiple myeloma comparing to healthy controls, in order to identify possible significant changes of cytokines concentration. *Results.* Our study showed a significantly increased level of TNF-α in the peripheral blood of multiple myeloma patients (28.21±17.08 pg/mL) compared with those obtained in healthy controls (4.6±14.6 pg/mL) (P<0.05). IL-1 and IL-6 proinflammatory cytokines were found in all multiple myeloma patient's serum and the value were higher than in healthy controls. IL-6 in multiple myeloma patients serum was 22.3±10.7 pg/mL compared with the healthy group 6.76±3.78 pg/mL (P<0.05), and IL-1 was 4.9±2.3 pg/mL in multiple myeloma *vs.* 2.24±0.94 pg/mL in healthy patients. *Conclusions.* Our study results show that IL-1 and IL-6 are involved in the multiple myeloma pathogenesis and argue for the existence of a systemic inflammatory response. TNF-α cytokine is constantly present in the peripheral blood of multiple myeloma patients.

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THROMBOPHILIA STUDY IN MULTIPLE MYELOMA (MM) PATIENTS

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Background. Thrombosis correlates significantly with MM. The role played by coagulation system in MM thrombosis is unclear. This may partly be due to lack of tests that truly reflect the balance between mechanisms that promote or inhibit the coagulation *in vivo*. Recently, a test is available for routine measurement of total thrombin potential (ETP) from Dade Behring. *Aim.* Comparison of ETP parameters with other coagulation markers like: - Antithrombin III, protein C, protein S, lupus anticoagulant, plasminogen, a2-antiplasmin, APCR, homocystein- between controls and MM patients. *Methods.* 18 consecutive samples of MM patients (7 men, 38.9% and 11 women 61.1%) and 30 control group samples were studied for ETP parameters, Antithrombin III, protein C, protein S, lupus anticoagulant, plasminogen, a2-antiplasmin, APCR, homocystein. We used the chromogenic method on the fully automated Behring Coagulation System (BCS) for the measurement of thrombin generation parameters. It should be noted that no patient received thalidomide for which is known to increase the likehood of thrombosis in MM patients. *Results.* Table. *Conclusions.* 1.ETP had not statistical significance between the control group and MM patients. 2.Acquired APCR is known to be a predisposing factor for thrombosis but was found in just one case. The ATIII, protein S, the LA and homocysteine had statistically significant differences between MM patients and controls. 3.Thrombophilic alterations in coagulation were not found in ETP parameters of our study. Despite that, ETP measurements may be useful as a simple method to identify patient of MM at risk for thrombosis and in whom the prophylactic anticoagulation may be necessary in conjunction with therapies such as thalidomide, which increase the risk of thrombosis.

Table.

Parameters	Controls	Patients	*P* Value
INR	0,9	1,3	0,001
APTT	30,6	42,7	0,0001
Fibrinogen	404	552	0,008
D-Dimer	0,3	0,7	0,005
Tlag	19,3	24,2	0,004
Tmax	54,4	91,2	0,0001
Cmax	123,5	94,1	0,0001
ETP	394,7	369,6	0,274
ATIII	100	89,2	0,0001
C	105	111,6	0,137
S	110	70	0,0001
LA	1	1,6	0,0001
plasminogen	107	109	0,559
a2antiplasmin	105	104	0,8
homocystein	10	16	0,0001
APCR	1	1,1	0,2

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DIFFERENCE IN SERUM CYTOKINES PATTERN BETWEEN SYSTEMIC AND LOCALIZED AL AMYLOIDOSIS

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Background. AL amyloidosis, a plasmacellular discrasia, is characterized by the deposition of proteic fibrils that infiltrate tissues leading to organ failure. Amyloid deposition can be systemic, affecting many different organ systems, or organ-specific. *Aims.* The aim of this study is to evaluate serum cytokines levels in patients with systemic or localized AL amyloidosis at presentation to find out potential differences. *Methods.* Blood samples were collected from 6 patients with systemic amyloidosis and from 4 patients with localized amyloidosis. Cytokine panel used included IL-10 and VEGF. Mann-Whitney test was used to compared results. IL-10 and VEGF were also tested in a group of healthy people as control. *Results.* Results showed that IL-10 level was significantly higher in the serum of patients with localized disease compared to the group of patients with systemic amyloid involment (P=0.006), VEGF was instead significantly increased in systemic disease group (P=0.027). *Conclusions.* Results seems to suggest a difference in serum cytokine pattern between AL systemic and localized amyloidosis. In particular, in localized disease T-regulators activity seems to be more efficient then in the systemic form in which we observed an increase in VEGF values maybe related to a more advanced neoplastic neoangiogenesis.

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THE COMPARISON OF SERUM AND PLASMATIC LEVELS OF SELECTED BIOMARKERS IN MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE AND MULTIPLE MYELOMA.

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Background. Multiple myeloma (MM) is characterized by proliferation of malignant plasma cells that accumulate in the bone marrow and often produce a monoclonal immunoglobulin. Angiogenesis activators include vascular endothelial growth factor (VEGF) and plasma concentrations of cytokines such as Tnf-a and Il-6 play a role in MM pathogenesis. They have been shown to stimulate the proliferation of malignant plasma cells as well as nonmalignant stromal cells. *Aims.* The aim of our study was to evaluate different expression of serum and plasmatic concentration of Tnf-α, Il-6 and VEGF in MM and MGUS patients. *Methods.* The levels of VEGF, TNF-α and Il-6 were detectable in 44 MM patients,48 MGUS patients and 14 healthy controls from 2004 today. The detection of the cytokine levels was performed by ELISA technique. Statistical analysis was perfomed using STATA program, t-test and Spearmen test. *Results.* M/F was 21/23 in MM patients, their median age was 69.5 yr (range 41-88). 18/44 (41%) patients showed a monoclonal component IgG/k, 11/44(25%) IgG/l, 4/44(9%) IgA/k, 7/44(16%) IgA/l; 4/44(9%) micromolecular type. 29/44(65%) patients presented comorbidities. The most was in advanced disease (Salmon and Durie stage III). Median concentration of B2 microglobuline was 3.5 mg/dL (range 1-16). 15/44(34%) patients presented abnormal cariotype. 24/39(62%) patients were in first; 10/39(25%) in second and 5/39(13%) in third/fourth lines therapy. M/F was 27/21 in MGUS patients, their median age was 68 yr (range 39-85). 5/48(10%) patients showed a monoclonal component IgA/k, 2/48(4%) IgA/l, 19/48(40%) IgG/k, 15/48(15%) IgG/l; 6/48(13%) IgM/k, 1/48(2%) IgM/l. 39% of this group had comorbidities. There weren't different expression of serum and plasmatic TNF-α and Il-6 between MM and MGUS patients. Only VEGF serum concentration was statistically significant into two study groups (P=0.012). Il-6 levels showed a progressive increase from healthly controls to MM patients. In MM patients we found statistically significance differences between serum and plasmatic medium concentration of VEGF (P<0.001), TNF-α (P<0.02) and Il-6 (P<0.005). We found a significant correlation between the concentration of VEGF and Il-6 and the number of platelets in those patients analyzed. *Conclusions.* Plasma and serum concentrations of these cytokines are not conclusive as to their relative importance in pathogenesis and progression from MGUS to MM. Since median concentration of all cytokines were bigger in serum, we think that this analysis could be more sensibility. Neverthless these data need confermation on a larger cohort of patients.

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THE INTRODUCTION OF SERUM FREE LIGHT CHAINS ASSAY ON THE DIAGNOSIS AND SCREENING PROTOCOLS FOR MONOCLONAL GAMMOPATHIES IS ESSENTIAL TO AN EARLY DIAGNOSIS

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Background. Monoclonal free light chains are homogeneous populations of kappa (κ) or lambda (λ) immunoglobulin light chain molecules produced by malignant B-cells clones. They are important tumour markers for identifying and monitoring patients with monoclonal gammopathies like multiple myeloma (MM), solitary plasmacytoma, monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma. The identification and quantification of monoclonal immunoglobulin in plasma cell disorders is based on electrophoresis, immunofixation and nephelometric measurement of immunoglobulins. The use of serum κ/λ FLC ratio is an emergent method that is becoming very important for the diagnosis of a plasma cell dyscrasia. An altered serum κ/λ ratio is a marker of monoclonality. *Aims.* We present a case that only serum protein electrophoresis (SPE) and free light chain (FLC) tests was necessary for efficient and quickly inicial diagnostic screen for monoclonal gammophaty. *Methods.* Serum free light chains (Freelite. The Binding Site) and immunoglobulins were measured by nephelometry in a BNII (Siemens). Serum and urine electrophoresis and serum and urine inmunofixation (SEBIA). *Results.* This case reports a 41 years old male who was admitted to the rheumatology services of hospital with a strong thoracic pain. He presented four vertebral fractures in dorsal region. Due to these lesions and to low total proteins and immunoglobulins (Total proteins: 6.0 g/dL and IgG = 307 mg/L; IgA = 30,4 mg/L; IgM = 10,1 mg/L) a MG (monoclonal gammopathy) suspicion was raised even he was a young patient. SPE not present monoclonal component. Even if the SPE was negative the results from the radiologic studies and immunoglobulins could induce the clinician to the suspect of a multiple myeloma or other monoclonal gammopathy. sFLC were requested and extremely high values were found (κ = 2710 mg/L; λ = 0,35 mg/L ratio κ/λ = 7742,86). Serum and urine immunfixation were then performed and a weak free K light chain was detected in both serum and urine IFE corresponding to the sFLC *Results.* After these results a bone marrow biopsy was requested and a 61% of plasma cells infiltration was found. Phatologic immunophenotype ($CD56^+/CD19^-$) compatible with MM. *Conclusions.* Even if SPE is the gold standard for the screening of monoclonal gammopathies, the sFLC measurement together with the ratio allows us to suspect a monoclonal gammopathy without recurring to expensive and invasive techniques for the patient achieving a quick and effective diagnosis. The high specificity and sensibility of serum free light chains allow us an early detection of monoclonal gammopathy or the exclusion of pathology.

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MULTIPLE MYELOMA PRESENTING AS SEVERE RENAL FAILURE: RELATED AND PROGNOSTIC VARIABLES IN A COHORT OF 391 PATIENTES

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Background. The multiple myeloma (MM) presenting as severe renal failure (SRF) has reduced the chances of treatment and survival. The detection of prognostic variables can provide decision-making but may vary by study population. *Aims.* To assess variables associated whit SRF and its prognostic utility regarding recovery of renal function in newly MM diagnosed patients. *Methods.* We retrospectively evaluated from the cohort of MM patients diagnosed in our department (1980-Jaunary to 2008-Mars) those affected by concomitant SRF defined as serum creatinine equal o superior to 5 mg/dL. We assessed the variables usually considered in this context for the remaining patients, and within the IRS group, the prognostic variables of renal function recovery. Diagnostic criteria for MM and response: those the Chronic Leukemia-Myeloma Task Force. Statistical *Methods.* univariate analysis using chi-square, Student t test and multivariate analysis by Cox binary logistic regression. *Results.* There were 376 patients with symptomatic MM; median age 68 years (31-93); monoclonal component (MC) (%): G (47), A(31), light

chains /others (57%); urinary MC present in 60%. Durie-Salmon stage (DS) (%): 1(15), 2 (25), 3 (60). DS A: 75%; B: 25%. ISS in 202 patients(%): 1 (29); 2 (36,5); 3 (35,5). Thirty-three patients (8,8%) had IRF at diagnosis; median creatinine 7,78 mg/dL (5-20). Urinary MC was detected in 88%; There were 13 (39%) light chains MM. In the univariate analysis, significant variables in relation to SRF were: ISS, MM, light chains MM, performance status, Hb, bone marrow plasmacytosis , amount of serum MC, calcemia and b-2 microglobulin. In multivariate analysis, however, the only variable that was significantly associated with SRF was hemoglobin. Eleven of the 33 patients required hemodialysis. First line chemotherapy was: MP(melphalan-prednisone) (6); VAD(vincristine-doxorrubicin-dexametasone) (16); other chemotherapy (11). In relapse or progression were used too, from 2003, thalidomide and from 2004 and 2006, bortezomib and lenalidomide respectively. The response rate was 28% (1 RO IF-, 7 RP); no response 21 (72%)patients, not evaluable 4 (12%). Fifteen patients (45%) recovered renal function (creatinine <2,5 mg/dL) at 3 months and 12 (36%) died within three months. Seven(21%) lived more than 2 years and two patients are living more than 7 years. We finally assessed the variables related to recovery of renal function or death before three months. In the univariate analysis was significant only hemoglobin and ISS and urinary MC were in the borderline. In multivariante analysis, hemoglobin was also the only significant variable.*Conclusions.* Our resuts confirm the poor prognosis of the SRF concomitant with the diagnosis of MM, although a subset of these patients can achieve long survival. The fact that hemoglobin has probed the only significant variable both in relation to the frequency of SRF and its reversibility could be explained because it is related to the MM and the severity and previous duration of renal failure.

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LIGHT CHAIN DEPOSITION DISEASE (LCDD) VERSUS AL AMYLOIDOSIS: DIFFERENCES IN NATURAL HISTORY AND TREATMENT?

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Background. LCDD is characterized by granular deposition of monoclonal immunoglobulin light chains and clinical presentation can mimic AL amyloidosis. Published descriptions of the natural history and treatment of LCDD are scanty. *Aims.* We wished to gain a better understanding of the differences between LCDD and AL amyloidosis, including natural history, response to treatment and overall survival. *Methods.* We conducted a retrospecive analysis of our experience at a single centre of LCDD, focusing on organ involvement, types of and responses to treatment and overall survival. *Results.* 24 patients had a histological diagnosis of LCDD. Another 6 patients with presumed LCDD were excluded from analysis as electron microscopy was not performed or unsuccessful. Males outnumbered females 2.4:1, and deposits were kappa class in 79% (19/24). Median age at diagnosis was 55 years (range 36-72). 2 patients had a bone marrow plasmacytosis >30%, and 5 patients had concomitant AL amyloidosis. All patients presented with renal involvement, and diagnosis was made on renal biopsy in 96%. At presentation all had haematuria, 50% with nephrotic range proteinuria, and 96% had marked hypertension. 7 patients had endstage renal failure (ESRF) at diagnosis. There was clinical evidence of cardiac, liver, gastrointestinal and skin involvement in 4, 4, 3, and 1 cases respectively. One patient had a factor X deficiency. Chemotherapy was administered to 96% of patients, including high dose steroids in 22 patients, CTD(a) in 14, ASCT in 5, VAD in 3, Mel Dex in 3, and bortezomib/dex in 2. Complete clonal responses (CR) were achieved in 3, and partial responses in 9 out of 19 evaluable patients. Median follow up was 40 months (range 1-91) with 18 patients alive at censor. 7 patients progressed to ESRF in a median of 47 months. Improvement in renal function was seen in 5 patients, defined as improvement by a CKD grade for >6 months (4 patients CKD V to IV; one CKD III to II) including 2 patients treated with short courses of steroids only - all had >90% reduction in light chain values. One patient, in CR post-ASCT, had a kidney transplant with good graft function at 10 months. Median projected overall survival was 7.5 years. Adverse prognostic features were heart or liver involvement and poor response to chemotherapy. *Conclusions.* LCDD patients are younger, with more kappa light chain involvement, hypertension and haematuria than patients with renal AL amyloidosis. Prolonged stabilisation or even improvement of renal disease can be seen when clonal disease is suppressed. An international registry would help determine optimal treatment and the role of renal transplantation.

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EFFICACY OF THALIDOMIDE-BASED AND BORTEZOMIB-BASED REGIMENS IN THE FIRST RELAPSE OF 146 MULTIPLE MYELOMA PATIENTS: A SINGLE CENTRE EXPERIENCE

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Background. The introduction of novel agents, such as immunomodulatory drugs and proteasome inhibitors, has substantially changed the treatment paradigm of multiple myeloma (MM). *Aims.* In this report, we describe the outcome of a cohort of 146 patients (pts) with the first relapse of MM treated with thalidomide-based and bortezomib-based regimens with aims to compare the efficacy of these therapies. *Methods.* Eighty-two percent of pts (119/146) had stage III according to DS, 17% (26/146) II and 1% (1/146) I, clinical stages according to ISS were the following: stage 1 in 44% of pts (64/142), stage 2 in 31% of pts (44/142) and stage 3 in 24% of pts (34/142). Renal insufficiency was presented in 14% of pts (19/146). Median age was 63 years (range: 33-84). Thalidomide-based (T) regimens were used in 99 pts; thalidomide + alkylating agent + dexamethasone: 86 cases (86%), thalidomide + dexamethasone: 5 cases (5%), alone thalidomide: 8 cases (8%). The daily dose of thalidomide was 100-200 mg. Bortezomib-based (B) regimens were used in 47 pts; bortezomib + alkylating agent + dexamethasone: 20 cases (42%), bortezomib+alkylating agent: 21 cases (45%), alone bortezomib: 6 cases (13%). Bortezomib was used in standard dose 1.3 mg/m^2 intravenously on days 1, 4, 8, 15. The cycle was repeated on day 21-28 for up to 8 cycles or until progression. Median of T and B treament cycles was 5, range 1-8. No statistically significant differences were observed between the T and B groups in baseline clinical parameters including age, clinical stages according to ISS and DS staging systems, the type of M-protein and presence of renal impairment. The first-line therapy was comparable for both T and B groups and it is not include novel agents. *Results.* In T group, overall response rate (ORR) was 56% (52/93), 12% of pts (11/93) achieved the complete response (CR), 20% of pts (19/93) were in very good partial response (VGPR), 24% of pts (22/93) in partial response (PR), 6% of pts (6/93) had minimal response (MR) or stable disease (SD) and 38% of pts (35/93) had progression of disease. Median time to progression (TTP) from the start of relapse treatment was 15.1 months, median overall survival (OS) was 30.5 months. In B group, ORR was 45% (17/38), 5% of pts (2/38) were in CR, 16% of pts (6/38) were in VGPR, 24% of pts (9/38) in PR, 18% of pts (7/38) had MR or SD and 37% of pts (14/38) had progression of disease. Median TTP from start of relapse treatment was 14.5 months, median OS was 34.4 months. *Conclusion.* The thalidomide-based and bortezomib-based regimens are effective in treatment of the first relapse MM with ORR 45-56%. Medians of TTP and OS are similar for both treatment groups, combination of novel agent with other drugs (dexamethason and/or alkylating agent) seems to be very useful. However, the significance part of patients (37-38%) had progression on this treatment and other treatment strategies are needed.

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ANALYSIS OF HEAVY/LIGHT CHAINS PROVIDES A RAPID, SENSITIVE AND ACCURATE ALTERNATIVE TO SERUM PROTEIN ELECTROPHORESIS AND IMMUNOFIXATION FOR PARAPROTEIN DETECTION AND QUANTIFICATION

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Introduction. Detection and quantification of monoclonal serum proteins by traditional serum protein electrophoresis (SPE) can be difficult and requires skilful interpretation. This is particularly true for IgA monoclonal proteins whose migration on the gel can often be obscured by co-migrating proteins. Immunofixation (IFE) can alleviate some of these issues, however it is non quantitative and can be time consuming. Serum free light chain (FLC) analysis can be useful, although international guidelines recommended a concentration of greater than 100mg/L for patient monitoring. Novel nephelometric assays have now been produced which quantify IgAκ and IgAλ in serum. Quantification of these proteins enables the calculation of an IgAκ / IgAλ (HLC) ratio which has been reported to have a greater sensitivity than IFE. Here we report the evaluation of these reagents in monitoring a cohort of IgA patients through the course of their disease and compare their utility to SPE, IFE and FLC measurements. *Materials and Methods.* Sequential samples from 53 IgA (29 IgAk / 24IgAl)

multiple myeloma patients were analysed (mean number of patient samples= 4, range 1-12). SPE and IFE were performed using SEBIA Hydrasys according to manufacturer's instruction. FLC and heavy/light chain analysis was performed on a Siemens Dade Behring BN™II analyser. *Results/Discussion.* 29/53 patients had presentation samples available; in 29/29 cases an abnormal HLC ratio correctly identified the monoclonal protein, matching IFE *Results.* In 14/53 patients the monoclonal protein was obscured by other proteins and accurate quantification was not possible; the HLC ratio could be used to monitor the patients through the course of their disease in all cases. In an additional 10/53 patients HLC ratios identified monoclonal disease which was below the sensitivity of SPE in 5/10 patients HLC identified monoclonal proteins below the sensitivity of IFE. 23/53 patients had FLC levels greater than 100 mg/L. In 3/23 patients the HLC ratio remained abnormal after the FLC ratio had normalised, conversely in 4/23 patients the FLC ratio remained abnormal after the HLC ratio had normalised. In 16/23 patients both assays had similar sensitivities. At relapse 5/53 patients the HLC ratios became abnormal before any other test, in 3/5 cases the abnormal ratio correctly identified early disease progression (75-282d). In 2/5 patients the disease progression occurred >1000d after the ratio had become abnormal. *Conclusions.* Abnormal HLC ratios correctly identified monoclonal proteins at presentation. Furthermore, HLC ratios quantified monoclonal proteins, even when they were obscured by other proteins on SPE. It is necessary to use both FLC and HLC in patient monitoring. Further work is needed to clarify what constitutes disease progression using the HLC ratios.

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THE EFFICACY OF BORTEZOMIB AND BORTEZOMIB-CONTAINING REGIMENS FOR TREATMENT OF RELAPSED/REFRACTORY MULTIPLE MYELOMA IN REAL CLINICAL PRACTICE

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Background. Bortezomib is currently considered as the standard of care for patients with relapsed/refractory multiple myeloma (R/R MM). However, there are few reports of outcomes with bortezomib treatment in regular clinical practice. *Aim.* To assess the efficacy of bortezomib alone and in combination regimens for treatment of patients with R/R MM at the Moscow Regional Research Clinical Institute. *Methods.* The study involved 101 patients with R/R MM (48 male and 53 female) aged from 34 to 77 years (average, 54 years). The average pretreatment time was 22.1 months and the average number of previous treatment cycles was 8.2 (MP; M-2; VAD). The study included 51 patients (50.4%) who had other pathologies: chronic renal failure and dependence upon hemodialysis, intraspinal plasmacytoma, lower extremity paresis, refractory anemia, or leukemic myeloma. Patients were randomized into four groups (V1-V4) to receive a bortezomib-containing regimen with reduced intensity of induction treatment: V1, (n=27) bortezomib-1.3 mg/m^2 on days 1, 4, 8 and11 of 31-d cycle; V2, (n=22) bortezomib-1.3 mg/m^2 on days 1, 4, 8 and 11; melphalan 20 mg/m^2 orally on the 2nd day of 56-d cycle; V3, (n=20) bortezomib-1.3 mg/m^2 on days 1, 4, 8 and 11; melphalan 9 mg/m^2 orally on days 1-4; prednisone 60 mg/m^2 on days 1-4 of 56-d cycle; V4, (n=32) bortezomib-1.3 mg/m^2 on days 1, 4, 8 and 11; melphalan 9 mg/m^2 orally on days 1-4; cyclophosphan i.v. 250 mg/m^2 on days 1-7; prednisone 60 mg/m^2 orally on days 1-4 of 60-d cycle. Treatment efficacy was assessed using the European Bone Marrow Transplantation (EBMT) criteria: time to progression; overall response rate (ORR=CR+nCR+PR); and overall survival (OS). *Results.* Patients received an average of 6.5 induction cycles. The following results were obtained: V1, ORR = 70.3% (CR 18.5%; nCR 14.8%; PR 37%); V2, ORR = 67.4% (CR 9.0%; nCR 13.0%; PR 45.4%); V3, ORR = 90.0% (CR 10.0%; nCR 0%; PR 80.0%); and V4, ORR = 62.3% (CR 21.8%; nCR 21.8%; PR 18.7%). There was no significant difference in OS between the four regimens. Since bortezomib was used in all four regimens, treatment efficacy was also assessed for the whole group of 101 patients. An objective response was obtained in 71.1% (CR 15.8%; nCR 13.8%). Progression was observed in 7.9% of patients within 6 months, and in 20.7% of patients within 20 months. Median OS estimated with the Kaplan-Meier method was 8.6 years (n=101). No cases of myelotoxicity grade III/IV were registered. Grade III polyneuropathy was observed in two patients. *Conclusions.* This study showed high efficacy and acceptable toxicity for bortezomib-containing reduced intensity regimens when used in a daily clinical practice for patients with R/R MM, including those who had been heavily pretreated or who had complicated comorbidities.

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CLINICAL CHARACTERISTICS OF BICLONAL GAMMOPATHIES REVIEW OF 101 CASES

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Introduction. Biclonal Gammopathy of Undetermined Significance (BGUS) is characterized by the simultaneous appearance of two narrow peaks, suggesting the existence of two monoclonal antibodies. The incidence is about 1% of all monoclonal gammopathies. The pathogenesis of BGUS is unknown, but several possibly related environmental factors have been identified. The type of monoclonal component (MC), level of uninvolved immunoglobulins (UI), Bence Jones proteinuria, light chains isotype, erytrosedimentation rate (ESR), percentage of bone marrow plasma cells are prognostic factors mentioned in the literature for the monoclonal gammopathies in defining the risk of malignant transformation. This study was performed in order to evaluate the baseline characteristics of a BGUS population and to determine the role of the same prognostic factors for the biclonal gammopathies's progression to myeloma. *Patients and Methods.* We performed a retrospective study of 101 consecutive patients observed between 1999 and 2009. Males were 53 (52 %) and females were 48 (48 %). Patients were divided in three groups of age: younger than 50 years (5 %, median 46y, range 38-49y), between 50-70 years (42 %, median 65y, range 53-70y) and older than 70 years (53 %, median 79y, range 71-92y). Clinical and laboratory features was selected for primitive BGUS so lymphoproliferative disorders, infections and solid tumors were excluded. Patients' characteristics baseline are shown in Table 1.

Table 1.BGUS patients' beseline characteristics (n=101).

Variable		N. (%)	Variable				N. (%)		
Gender	M / F	48(48) / 53(52)	Type of light chains			k/λ	50 (49)		
Age (y)	<50	5 (5)				k/k	33 (33)		
	50-70	42 (42)				λ/ λ	18 (18)		
	>70	54 (53)	Bence Jones	negative	positive	NE	84(83)	13(13)	4(4)
Isotype	IgG/IgA	38 (38)	[UI] (mg/dL)	normal	reduced	NE	41(41)	26(26)	34(33)
	IgG/IgM	28 (27)	PC (%)	≤ 5	>5	NE	22(22)	17(17)	62(61)
	IgG/IgG	17 (17)	Albumin (g/dL)	≥ 3.5	<3.5	NE	91(90)	10(10)	0
	IgM/IgA	6 (6)	ß2microG(mg/L)	≤ 2.5	>2.5	NE	44(44)	10(10)	47(46)
	IgM/IgM	8 (8)	ESR (mm/h)	≤ 15	> 15	NE	34(33)	46(46)	21(21)
	IgA/IgA	2 (2)	UI = uninvolved immunoglobulins; PC = plasmacells in bone marrow; ESR = erytrosedimentation rate NE= no evaluated						
	IgM/k free	2 (2)							

The median follow-up in no developed no complicated BGUS was 47 months (range 1-210). Two patients was excluded for the cancer appearance after a 24 and 29 months follow-up (lung and prostate respectively).Three patients (3 %) progressed to myeloma and their characteristics are shown in Table 2. The median follow-up was 252 months. *Conclusions.* Although the clinical features of biclonal gammopathies are similar to those of monoclonal gammopathy, this subject is of importance because of the lack of clinical data in the literature. Three progressed BGUS aren't many for conclusions so further a long term studies are ongoing to examine the natural history of BGUS using a prospective approach and a larger patients' cohort to identify variables associated with the progression, the rate of progression, the progression free survival, the overall survival and the type of myeloma evolution (monoclonal or biclonal myeloma).

Table 2. MM patients' characteristics (n=3).

Variable		N. (%)	Variable				N. (%)		
Gender	M / F	2 (67) / 1 (33)	Bence Jones	negative	positive	NE	2 (67)	1(33.3)	0
Age (y)	> 70	3 (100)	[UI] (mg/dL)	normal	reduced	NE	2 (67)	1(33.3)	0
Isotype	IgG/IgG	1 (33.3)	PC (%)	≤ 5	>5	NE	0	3(100)	0
	IgG/IgA	1 (33.3)	Albumin (g/dL)	≥ 3.5	<3.5	NE	3(100)	0	0
	IgM/IgA	1 (33.3)	ß2microglob(mg/L)	≤ 2.5	>2.5	NE	3(100)	0	0
Type LC	k/λ	2 (67)	ESR (mm/h)	≤ 15	> 15	NE	3(100)	0	0
	λ/ λ	1 (33)	UI=uninvolved Ig; PC =plasmacells ;ESR=erytrosedimentation rate; NE=no evaluated						

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CLINICAL VALUE OF ABSOLUTE LYMPHOCYTE COUNTS BEFORE BORTEZOMIB-DEXAMETHASONE THERAPY IN RELAPSED MULTIPLE MYELOMA PATIENTS

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Background. High absolute lymphocyte count (ALC) at diagnosis is known as a surrogate marker of host-immunity with favorable prognosis in newly diagnosed Multiple myeloma. Bortezomib is an effective proteasome inhibitor in relapsed multiple myeloma (MM). Recent studies have shown tumor sensitization and enhanced cytotoxicity of bortezomib. *Aims.* To evaluate whether high ALC before bortezomib treatment would contribute to tumor sensitization and activated cytotoxicity of bortezomib in relapsed MM. *Methods.* Ninety-seven relapsed MM patients who were treated with bortezomib-dexamethasone (Vel-Dex) therapy were analyzed in the present study. Median follow up duration was 21 months and median age was 61 years. *Results.* Complete response (CR) and very good partial response (VGPR) after 2 cycles of Vel-Dex therapy were higher in the high ALC group (≥ 1.1×10^9/L) than in the low ALC group (CR+VGPR, 50.0 % in the high ALC group *vs.* 10.4 % in the low ALC group, P=0.001), and stable (SD) was lower in the high ALC group than in the low ALC group (SD, 11.8 % in the high ALC group *vs.* 44.8 % in the low ALC group, P<0.001). In univarate analysis, the low ALC group before therapy was associated with shorter PFS (HR, 2.780; 95% CI, 1.703-4.536, P<0.001). Multivariate analysis revealed that the low ALC group before therapy represented an independent predictive factor for PFS (HR, 1.937, 95%CI, 1.168-3.212, P=0.010). *Conclusion.* Low ALC just before Vel-Dex therapy was associated with poor prognosis for the patient with relapsed MM.

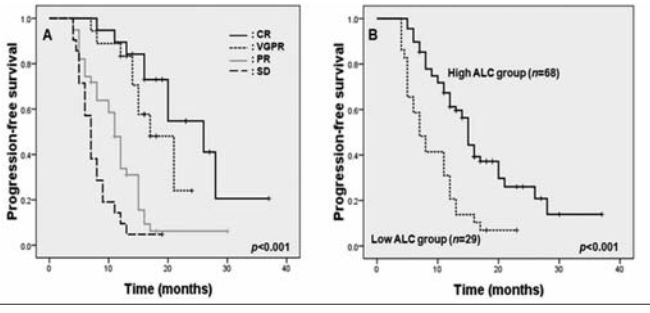

Figure.

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LONG SURVIVAL RELATED VARIABLES IN A COHORT OF 391 PATIENTS WITH MULTIPLE MYELOMA

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Background. It is known that a low rate of patients with Multiple Myeloma (MM) reach a long survival, not conditioned to a specific type of therapy.The factors related to it can vary depending on the environment for recruitment of the patients. *Aims.* To asses in a series of unselected patients with symptomatic MM treated with chemotherapy(CHT), the existence of initial variables related to long survival, together with the impact of response rate at 3 and 12 months of starting treatment. *Methods.* Retrospective study of the MM patients cohort treated in our Unit (1980-January to 2008-Mars). Length of observation time(OT): since the start of chemotherapy until death or 2010-January. Assessed groups: patients with more than 10 years survival or diyng before that interval. Diagnostic and response criteria according to Chronic Leukemia-Myeloma Task Force. Staging by Durie-Salmon and ISS.Delivered CHT was variable, depending on periods(MP; VMCP-VCAP/VBAP; M2 ; VAD).ASCT was performed since 1994. Thalidomide, bortezomib or lenalidomide had been recently incorporated as salvage. Initial clinical and analytical variables were compared between both groups; and moreover the rate of at least partial response at three months and progression earlier than 12 months. *Statistical methods.* Chi-square, Fisher exact test, Student t and Cox binary logistic regression meodel. *Results.* 327 of 391 patients were included in the study by exclusion of asymptomatic and treated patients with less than 10 years of survival. Median age: 68 (31-93) years.Gender(M/F): 170/157. Types of monoclonal component(MC)(%): G (46); A (31); light chain and other(23). Median serum MC: 3.6 g / dl (0,3-10); urinary MC present in 60%. Durie-Salmon staging (%): I (13), II (23), III (64); A/B: 75/25. ISS (154 patients)(%): 1 (21), 2 (40) 3 (39). Median β-2 microglobulin(B2M): 4 mg / L (0,8-70). Median bone marrow plasmocytosis(BMP): 40% (3-100). 23 patients (7%) survived 10 or more years with median OT of 12 years (10-24). The remainder of patients died with median survival of 1.6 years (0.02-9.6). ASCT were performed in 44 patients (double in 12), 9 of them in the long survival group (double in 4). In the long survival group it was significantly more frequent: good performance, favorable ISS and DS A stage; on the other side, age, BMP, calcemia and serum MC were significantly lower; on the contrary hemoglobin and serum albumin were higher.We did not find significant difference in the proportion of patients with three months at least partial response, but progression at 12 months was lower in the long survival group (P:0.0015). However, in multivariate analysis the only significant variables were younger age (P:0.0008), lower serum MC (P:0.03) and BMP (P=0.01) in long survival group.Conclusion. Survival over 10 years in our symptomatic MM is significantly associated with younger age, and lower plasmacytosis and serum CM. These results can be interpreted as the consequence of tumor burden (BMP and serum MC) and the better tolerance and available treatments in younger patients.

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THALIDOMIDE AND LOVASTATIN AS A SALVAGE IMMUNOMODULATORY THERAPY IN PATIENTS WITH RELAPSED MULTIPLE MYELOMA

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Background. Lovastatin (LOV) and other inhibitors of HMG-CoA reductase, the rate-limiting enzyme of the mevalonate pathway, have been demonstrated to exhibit antineoplasmatic and proapoptotic properties in numerous *in vitro* studies involving myeloma cell lines including our own experiments. *Aims.* The aim of the study was to assess the influence of lovastatin addition on the treatment effects in patients with relapsed and refractory MM. *Methods.* We have treated 49 patients with TAL+DEX (TD) regimen and 42 patients with TAL+LOV+DEX (TLD) regimen. Patients received drugs orally in 28-day cycles. TAL was given from day 1 to day 28 each cycle of dose 100 mg daily. DEX was administered at dose of 20 mg daily in days 1-4 of each cycle. LOV was administered at a dose of 2 mg /kg in days 1-5 and 8-12 and at a dose of 0.5 mg/kg in days 15-28 of each cycle. Patients characteristics before treatment were as follows: the median age 59.3 years in TLD and 62.4 years in TD . All of them were ISS 2. 61% of patients IGG, 26% IGA, 7% light chain and 6% others. 76% were light chain kappa and 24% lambda. Median serum M protein level was 4.2 g/dL, bone marrow plasma cells 47%. *Results.* A clinical response, defined as a reduction of M-protein by 50% or more, was observed in 32 % of patients in TD group and 44 % in TLD group. CR and NCR were observed in TD 5% and 11% TLD respectively. In 47.9% TLD and 14.6% TD patients successful stem cell harvest was performed and mean amount of collected $CD34^+$ cells was 6.76×10^6 in TD and 8.26×10^6 in TLD. Successful autologous transplantation was performed in 3 patients of TD and 11 TLD. Overall survival (OS) in TLD group (median 53 months) was significantly longer than TD group (39 months). Similarly event free survival (EFS) was longer in TLD (median 27 months) than in TD group (23 months). We observed significant negative correlation between response and bone marrow infiltration (P<0.005). Short time to reduction of M-protein by 50% was connected with better response. Common side effects such as somnolence, fatigue and constipation were observed in about 18 patients in TLD and TD group. In 2 TLD and 1 TD patients we diagnosed deep vein thrombosis. In 5 five TLD patients moderate hypertransaminazemia was observed. No TLD treated patients were observed with the rise of myoglobine and troponine in the control blood examinations. In 2 TLD patients sinus bradykardia was observed. *Summary/Conclusions.* Our results suggest that addition of LOV to TAL and DEX improves the response rate as well as OS and EFS in patients with relapsed or refractory MM. Moreover it is possible to harvest stem cells and perform autologous stem cell graft in patients with relapsed or refractory MM treated with such regimen. The addition of lovastatin during the salvage therapy in the relapsed or refractory form of MM seems to be a very efficient and nontoxic.

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COMPARISON OF HUMORAL IMMUNE STATUS IN ELDERLY PATIENTS WITH MULTIPLE MYELOMA, WALDENSTROM'S MACROGLOBULINEMIA AND MONOCLONAL GAMMOPATHY OF UNDETERMINED SIGNIFICANCE

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Background. It is well known that patients with multiple myeloma (MM) have impaired humoral immunity, but the immune status of patients with Waldenstrom's macroglobulinemia (WM) and monoclonal gammopathy of undetermined significance (MGUS) has been less studied. *Aim.* The aim of this study was to compare humoral immunity to various common pathogens in elderly patients with these B-cell disorders. *Methods.* Seventy-nine persons ≥ 60 years of age were studied, with a diagnosis of MM (n=25), WM (n=16), MGUS (n=18) and healthy controls (n=20). Patients who had undergone stem cell transplantation were excluded. Serum samples were analyzed for antibodies to in total 26 bacterial, viral, fungal and protozoan pathogens. *Results.* A stepwise pattern of antibody levels was seen for staphylococci, pneumococci, tetanus and diphtheria with the lowest titres observed in the MM group followed by, in increasing order, WM, MGUS and healthy controls (P<0.001 for MM *vs.* controls). Whereas 65% of healthy controls were immune to tetanus, a mere 8% of MM patients had protective antibody levels despite previous immunization being reported by 56% to 83% of all study subjects. Long-term immunity to diphtheria was absent in all studied subjects (n=79) including healthy controls although 35% reported previous immunization. As to viral pathogens, a stepwise antibody pattern was seen for measles, mumps, rubella, and was most pronounced for varicella (P<0.0001 for MM *vs.* controls), with the lowest antibody titres seen among MM patients. Analyses of antibodies to the other Herpes viruses (HSV-1, HSV-2, CMV, EBV and HHV-6) on the other hand, revealed no differences between the study groups, and except for HSV-2, high levels of protection were seen. Antibody titres to Candida albicans, Aspergillus fumigatus and Toxoplasma gondii were particularly depressed in the MM group. The analysis of IgG antibodies to Borrelia burgdorferi revealed a strikingly high prevalence with 21 (27%) of the study persons defined as seropositive and no differences seen between the study groups. *Summary.* In conclusion, the multiple myeloma patients displayed by far the most suppressed humoral immunity among the patient groups studied. However, depressed humoral immunity against many infectious agents was also seen in patients with Waldenstrom's macroglobulinemia and monoclonal gammopathy of undetermined significance as compared to healthy controls. The most obvious immune impairment was found towards Staphylococcus aureus, pneumococci and varicella, while seroprotection to other Herpes viruses seemed to be retained in patients with these B-cell disorders.

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BIGGER IS NOT BETTER IN PBSC MOBILIZATION WITH CYCLOPHOSPHAMIDE FOR AUTOLOGOUS STEM CELL TRANSPLANT IN MYELOMA: A RETROSPECTIVE SINGLE-CENTRE AUDIT OF 1.5G/M² VS 3G/M² CYCLOPHOSPHAMIDE WITH G-CSF

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Background. High dose chemotherapy followed by autologous stem cell transplant in first CR is the standard of care for myeloma patients fit enough to undergo the procedure. However, mobilization methods vary with no generally accepted consensus. Cyclophosphamide with G-CSF is the most frequent mobilization chemotherapy in the West of Scotland: however, dose administered varies between 1.5 and 3 g/m² dependent on preference of referring physician. Only one previous relevant comparative study has been published (Hiwase *et al.,* Cytotherapy 9:539-547,2007), which suggested better mobilization efficacy with 3-4 g/m² cyclophosphamide compared with 1-2 g/m². However, due to a local impression of increased toxicity with 3 g/m² of cyclophosphamide without much difference in mobilization efficacy, we felt that a single-centre audit of our own experience would be useful. *Aims.* To compare mobilization failure rate and total CD34 dose collected following cyclophosphamide 1.5 g/m² *vs.* 3 g/m² both with G-CSF 5mcg/kg. *Methods.* All myeloma patients referred to BWOSCC clinical apheresis unit for collection of autologous stem cell collection over the period January 2007 to November 2009 were assessed. 83 patients received cyclophosphamide and G-CSF. Dose of stem cells collected, number of apheresis procedures per mobilization episode, and failure to achieve a good enough peripheral CD34 count to undergo apheresis were recorded. The Chi-Square and Mann-Whitney U statistical tests were used to determine significance. *Results.* See Table. There was a significant increase in complete mobilization failure (failure to undergo any apheresis) in the 3g/m² group. There was no significant difference between number of procedures required to collect stem cells in either group. The number of patients achieving a total dose sufficient for 2 transplants was similar in each group and there was no significant difference in the number of patients unable to collect enough stem cells for at least one transplant in either group. There were 5 patients in each group who developed neutropenic fever requiring admission and antibiotics, which was associated with subsequent failed PBSC mobilization in 4 of the 3 g/m² patients and 1 of the 1.5 g/m² patients. Neutropenic fever appeared more severe and prolonged in the 3 g/m² group, and we believe this to be the likely cause of the significantly higher rate of complete mobilization failure in this group. *Conclusion.* Contrary to the findings of Hiwase *et al.,* we found cyclophosphamide 1.5 g/m² with G-CSF as effective as 3g/m² in mobilizing enough cells for tandem transplant, and a significantly higher rate of complete mobilization failure in the 3g/m² group, associated with more severe toxicity in the form of neutropenic fever. In view of these results, we now recommend 1.5g/m² as the standard dose for our myeloma patients, particularly given the availability of non-chemotherapeutic stem cell mobilizing agents such as plerixafor for patients failing initial chemotherapy-based mobilization.

Table.

	3g/m2	1.5g/m2	
No of Patients	30	53	
Failed Mobilization	5	1	P<0.02
Median no. Procedures (Range)	2 (1-3)	2 (1-3)	
CD34 dose >6 (%)	18 (60)	34 (64)	
CD34 dose <2 (%)	4 (13.3)	4 (7.5)	
CD34 dose 2-6 (%)	3 (10)	13 (24.5)	
Neutropenic Fever (No of failures)	5 (4)	5 (1)	

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HIGH-DOSE OR INTERMEDIATE-DOSE CYCLOPHOSPHAMIDE PLUS GRANULOCYTE COLONY-STIMULATING FACTOR FOR PROGENITOR CELL MOBILIZATION IN PATIENTS WITH MULTIPLE MYELOMA

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Autologous PBSC transplantation is the standard care for patients with multiple myeloma (MM). Cyclophosphamide (CY) combined with granulocyte colony-stimulating factor (G-CSF) is commonly used to mobilize blood progenitor cells to support high-dose therapy in patients with MM. The optimal dose of CY in this setting is unknown. *Methods.* We have analyzed mobilization efficiency and need for supportive care in 81 patients (pts) with newly diagnosed myeloma previously treated with VAD or bortezomib-containing courses. The patients were mobilized either with high-dose CY (HD-CY, 6 g/m²) (n=61) or intermediate-dose CY (ID-CY, 4 g/m²) (n=20) plus G-CSF 5 mcg/kg/day. *Results.* Both regimens proved to be effective in the progenitor cell mobilization. In the HD-CY group, 30,5 (1,1-10⁶)×10⁶ CD34⁺ cells/kg, and in the ID-CY group 17,6 (2,5-22,7)×10⁶ CD34⁺ cells/kg (P=0,0001), were collected. At least 2×10⁶ CD34⁺ cells/kg were collected from 90,2% and 85% of the patients with a first apheresis respectively. Patients who are to have tandem autologous PBSC transplants require at least 4×10⁶ CD34⁺ cells/kg. This was achieved in 96,7% pts in the HD-CY group and in 95% pts in the ID-CY group. Only two pts in the HD-CY group (3,3%) failed to mobilize *vs.* none in the ID-CY group. The highest yield of peripheral

blood CD34+ cell was reached in the HD-CY group. The median number of aphereses performed in each group was 2 (range 1-6). The first day post CY infusion of first leukapheresis was between day 12 and day 20 (median day 15 in HD-CY group and 14,5 in ID-CY group). Pts mobilized with ID-CY plus G-CSF had less toxicity: lower frequency of febrile episodes (35 *vs.* 49,3%) and less need for supportive care including parenteral antibiotics (35 *vs.* 49,3%), platelet transfusions (25 *vs.* 59%, P=0,01). Also, nausea and vomiting were less frequent and less severe in patients receiving ID-CY. *Discussion.* While these regimens seem to be equally effective in terms to collect a total CD34+ cell number adequate for tandem autologous PBSC transplantation in newly diagnosed patients with MM, ID-CY+G-CSF is preferential because of more optimal resource utilization and more favorable toxicity profile.

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SOLUBLE UROKINASE-TYPE PLASMINOGEN ACTIVATOR RECEPTOR (SUPAR) IN PATIENTS WITH MULTIPLE MYELOMA (MM). RELATIONSHIP TO AMYLOIDOSIS, RENAL FUNCTION AND CLINICAL COURSE

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The urokinase-type plasminogen activator (uPA) system has been shown to be involved in tumour cell migration, angiogenesis and tissue remodeling. The expression of uPAR by malignant plasma cells and its soluble form (suPAR) may play a relevant role in plasma cells invasion, homing and clinical evolution. The study was undertaken to evaluate the serum concentration of suPAR in patients with MM in order to assess its relation to the amyloidosis, renal function and clinical course of the disease. We analyzed 70 patients treated in the Hematology Department of District Specialist Hospital of Legnica and Dept. of Haematology, Blood Neoplasms and Bone Marrow Transplantation, Wroc_aw Medical University, from 2007 to 2009. The group consisted of 40 females and 30 males aged 28-83 years (x=62,1), 37 (52,9%) recently diagnosed (group I) and 33 (47,7%) in the progression of the disease (group II), 6 pts of group I and 12 of the group II had amyloidosis. Renal failure developed in 15 pts. The control group comprised 10 healthy donors. Written informed consent was obtained. Blood samples (5 ml) for analysis were taken at diagnosis, or at the time of progression, the serum concentration of suPAR was determined by ELISA and expressed in pg/mL. Diagnosis of AL was based on finding in the biopsy of adipose tissue, deposits showing a typical green birefringence under polarized light. The serum concentration of suPAR in patients with MM ranged from 1450 to 22602, x= 3813, SD= 2949 and was significantly higher (P=0.0002) than in the control group: 1783-2732, x=2211, SD=345, the highest in 6 pts with LCD: 2399-22602, x=7453, SD=7659. In patients before chemotherapy suPAR concentration was insignificantly lower than those with progression of the disease: x=3567, SD=2027, and x=4577, SD= 4828, respectively. suPAR concentrations did not differ between patients with clinical stage I, II and III however patients with stage III revealed a tendency to higher suPAR concentration (P=0.06). In patients with renal failure suPAR concentration was significantly higher (P=0.01) than in patients with normal renal function, and were as follows: 1749-5922, x=4593, SD=2848 and 1449-22602, x=3600, SD=2965. In MM pts with amyloidosis, suPAR concentration was significantly higher than in group without this complicantion : 1449-22602 x= 5211, SD= 4784 and 1552 -11686, x=3328, SD=1170, P=0,0047. There were positive correlations between suPAR concentrations and number of plasma cells in bone marrow as well as serum free light chain and β-2-m concentrations and negative correlation between suPAR and albumin level (P=0,005). These results suggest that suPAR might provide additional information related to a more aggressive course disease and development of amyloidosis.

1450

NORMAL SERUM FREE LIGHT CHAIN RATIO AT PRESENTATION IS MORE COMMON IN IGA- κ MULTIPLE MYELOMA: A SINGLE CENTRE OBSERVATIONAL STUDY

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Background. Several studies have shown that baseline serum free light chain ratio (sFLCR) is an independent prognostic factor in patients with multiple myeloma and other plasma cell disorders. Baseline serum free light chain correlated with serum creatinine, lactate dehydrogenase, marrow infiltration by plasma cells and light chain type multiple myeloma in some studies. The relationship between baseline sFLCR at diagnosis and the different subtypes of multiple myeloma has not been reported to date. *Aims.* To analyse the baseline sFLCR in relation to the different subtypes of multiple myeloma and to assess the relationship between the baseline sFLCR and parameters of disease activity. *Methods.* All newly diagnosed multiple myeloma patients in our centre between June 2005 and January 2010 were included. The serum free light chain measurement was performed by immunonephelometry (Freelite assay; The Binding Site Ltd., Birmingham, UK). sFLCR was calculated as κ/λ (normal range 0.26-1.65). Clinical and laboratory parameters at the time of diagnosis were recorded. *Results.* 48 patients were included in this study. The male to female ratio was 1.4:1 with a median age of 73 years (range 45- 94 years). The majority of the patients (56%) had IgG multiple myeloma, 31% had IgA multiple myeloma and 13% were light chain only subtype. 44 patients had their sFLCR measured at diagnosis. All patients with IgG and light chain only multiple myeloma had an abnormal sFLCR at presentation. Interestingly 33% of IgA multiple myeloma patients in our study showed an absolutely normal sFLCR with a further 20% of patients showing only mild elevation in monoclonal light chain. Normal sFLCR correlated with negative Bence Jones proteinuria, heavy bone marrow infiltration (>45% plasma cell infiltration), and the ISS stage II - III. However, there was no observed relation of the baseline sFLCR to serum creatinine, calcium level or serum albumin. The median baseline sFLCR in IgA-κ group was 1.79 (range 0.5 - 310.6) in comparison to 31.34 (range 1.74 - 1791.75) in IgG-κ group. In contrast, IgA-λ and IgG-λ groups showed no difference in the median baseline sFLCR. *Conclusion.* Our study has shown, for the first time, that IgA-κ multiple myeloma patients commonly present with normal sFLCR. Our data suggests that sFLCR at presentation may be of less prognostic value in IgA-κ multiple myeloma subtype. This might also have an implication in the assessment of stringent complete response in this group of patients. Further analysis is required to assess the clinical course in this group of patients.

1451

BALANCING EFFICACY AND TOXICITY BY OBJECTIVE CHARACTERISTICS IN PATIENTS WITH MULTIPLE MYELOMA TREATED WITH NEW-DRUG COMBINATIONS

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Background. New-drug combinations have changed the outcome of Multiple Myeloma (MM) but their administration frequently induce severe complications leading possibly to early death and often prevent therapy continuation particularly in elderly patients. Therapy withdrawal with new-drug combinations (until 30-40%) and early mortality actually represent the major cause of unsatisfactory outcome in MM patients. In order to adapt treatment schedule, age, performance status and comorbidities are empirically considered. However, there is no objective methods to identify MM patients who will develop severe complications or will die because of induction therapy with novel-drug combinations. *Aim.* To assess the potential of objective factors in predicting complications in MM patients treated with new-drug combinations. *Methods.* Patient, disease and treatment related characteristics of the 29 patients who developed severe complications such as grade 3-4 infections, deep venous thrombosis (DVT), any other adverse event preventing therapy continuation or who died because of induction therapy were compared to those of 98 patients who did not. One hundred and twenty seven *de novo* MM patients enrolled in phase II and III prospective, controlled trials were included in this study. Patients were treated with thalidomide (83%), bortezomib (17%) or both (14%) in combination with steroids and chemotherapy. Median age was 71 years (range 45-71), 70% of patients were older than 65 years and 25% aged 75 years or above. Twenty eight percent of patients presented ECOG PS ≥2,21% IgA myeloma, 70% 2-3 ISS stage, 20% renal failure and 30% had at least one comorbidity. All patients received antibiotic and antithrombotic prophylaxis. *Results.* An event, such as above defined, occurred in 29 patients. Thirteen patients (10%) had an infection, 11 (8.5%) had DVT, 3 (2.5%) had infection and DVT, 3 (2.5%) died early and 7 (5.5%) discontinued therapy because of severe toxicity. Probability of severe complication was 23%. Univariate analysis selected age ≥75 years (P= 0.072), IgA myeloma (P= 0.021), monoclonal component >3 g/dL (P= 0.059), ISS stage (P= 0.088), β-2 microglobulin ≥3.5 mg/dL (P= 0.133), albumin <3.5 g/dL (P= 0.021), CRP > 3 mg/dL (P= 0.153) and platelets <130.000/μL (P=0.027) as predictive

factors of severe complications whereas PS, comorbidities, D-S staging, bone disease, renal failure, and therapy regimens were not predictive. Multivariate Cox regression analysis selected monoclonal component >3 g/dL plus platelets ≤130.000/μL (HR= 3.0; P= 0.004) and IgA myeloma (HR= 2.3; P= 0.032) as factors associated with an increased risk of severe complications. *Summary.* This retrospective study suggests that in MM patients disease characteristics, rather than those patient- or therapy-related should be taken in account to balance efficacy and toxicity when treatment with new-drug combinations is planned.

1452

PRECLINICAL AND PHASE I EVALUATION OF THE PHARMACOKINETICS, METABOLISM, SAFETY AND EFFICACY OF A 30-MINUTE INFUSION OF CARFILZOMIB

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Background. Carfilzomib (CFZ) is a novel, highly selective, irreversible tetrapeptide epoxyketone proteasome inhibitor. In Ph I-II trials single-agent bolus CFZ is active at 20-27 mg/ m^2 and well-tolerated in patients with relapsed and/or refractory (R/R) multiple myeloma (MM). *Aims.* Preclinical studies in Sprague-Dawley rats were conducted in order to characterize the pharmacokinetics (PK), pharmacodynamics (PD), and toxicity of CFZ administered as an intravenous (IV) bolus or 30 minute infusion. The effect of IV infusion of CFZ is also being evaluated in a Phase Ib/II study in patients with R/R MM to determine the maximum recommended dose, safety, efficacy, PK and PD. *Methods.* In rats, a dose of 8 mg/kg (48 mg/m^2) was administered as an IV bolus (~20 sec) or a 30 minute infusion. Plasma samples were taken for PK and whole blood and tissue (adrenal, heart, liver) samples were collected to determine the extent of proteasome inhibition. PK parameters were calculated using non-compartmental analysis and proteasome activity was measured using fluorogenic substrates. The Phase I trial is enrolling patients with R/R MM after ≥2 prior treatment failures or following induction and transplantation. CFZ is given as a 30 minute IV infusion on days (D) 1, 2, 8, 9, 15, and 16 of a 28-day cycle (C) until disease progression. Dosing in all cohorts is 20 mg/m^2 for C1 D1, D2 with subsequent escalation to 36, 45, or 56 mg/m^2. Responses by IURC are measured every C.

Table.

Best Response	Dose (mg/m^2)[a]	Prior Regimens	Time on Study (months)
VGPR	36	1	7+
PR	45	4	4.5+
MR	36	6	5
SD	36	1	5.4
	45	4	2.4+

[a] Patients received 20 mg/m^2 on C1, D1 and D2, with subsequent dose escalation to the dose specified.

Results. Despite a >10 fold decrease in maximum concentration, infusion of CFZ in rats resulted in equivalent levels of proteasome inhibition in blood and tissues (>80%), suggesting widespread tissue distribution. IV bolus administration of CFZ at 8 mg/kg resulted in 50% mortality and elevated blood urea nitrogen and creatinine levels in the surviving animals. Infusion of this dose did not cause mortality or significant changes in renal function. To date, 7 pts with R/R MM are enrolled in the Phase 1b infusion study; 4 remain active. Pts remain on study a median of 136 days (range 32-213). They received a median of 4.9 cycles of CFZ (range 1.1-7.6). Preliminary data show that CFZ is well-tolerated and has anti-tumor activity when administered as an infusion. Efficacy data are detailed in the Table. Common adverse events (AEs) with infusional CFZ included fatigue, headache, diarrhea, nausea, and constipation. There has been no worsening of baseline peripheral neuropathy, severe hematologic toxicities, or hepatotoxicity. Updated results will be presented. *Conclusions.* In rats, infusion of high dose CFZ is substantially better tolerated than IV bolus and resulted in similar levels of proteasome inhibition. In patients with R/R MM, single-agent CFZ as 30 minute IV infusion is both active and well-tolerated at doses ≥ 36 mg/m^2, with documented PRs achieved, early in the course of therapy. These data support ongoing evaluation of CFZ infusion in patients with MM, and dose escalation beyond 45 mg/m^2 has been initiated.

1453

CLINICAL CHARACTERISTICS AND PROGNOSTIC SIGNIFICANCE OF HEPATITIS VIRUS INFECTION IN PATIENTS WITH MULTIPLE MYELOMA AND ITS ASSOCIATION WITH CYTOGENETIC ABNORMALITIES

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Background. Long-term steroid treatment may lead to reactivation of hepatitis virus infection and therefore probably affect the treatment outcome of patients with multiple myeloma. However, reports regarding the impact of hepatitis virus infection in myeloma are limited. *Aims.* We assessed the prevalence and prognostic significance of viral hepatitis in myeloma patients. *Methods.* From January 2003 to December 2008, patients with confirmed diagnosis of multiple myeloma at Taipei Veterans General Hospital were consecutively enrolled. Hepatitis virus infection was determined by serology tests for hepatitis B and C virus. Clinical characteristics were recorded via chart review, and univariate and multivariate analysis were performed to investigate the significance of relevant parameters. *Results.* Totally 222 patients were enrolled and 68.9% (153/222) of them had completed studies for hepatitis virus. The estimated prevalence of hepatitis B and C virus infection was 11.1% (n=17) and 9.3% (n=14) respectively. Median age at diagnosis was 69.0 years (range, 29-91) and median follow-up was 19.3 months (range, 0.2-83.9). The characteristics of patients with or without hepatitis virus infection were similar, but viral hepatitis carriers were strongly associated with the occurrence of cytogenetic abnormalities (P=0.005). Hepatic adverse events did not differ in 2 groups except that one patient with chronic hepatitis B died of fulminant hepatitis. Overall survival of patients with hepatitis virus infection was significantly worse than that of whom without the infection (42.4 *vs.* 16 months, P=0.023). In multivariate analysis, older age, cytogenetic abnormalities, and beta-2-microglobulin of more than 3.5 μg/mL at diagnosis were independently correlated with shorter overall survival. *Conclusions.* Hepatitis virus infection exerts a poor survival impact on patients with multiple myeloma, which might be resulted from the association with cytogenetic abnormalities. Further studies to elucidate the relationship between viral hepatitis and chromosomal abnormalities in myeloma appear to be warranted.

1454

AGGRESSIVE APPROACH WITH BORTEZOMIB AND CYCLOPHOSFAMIDE, FOLLOWED BY AUTOLOGOUS STEM CELL TRANSPLANTATION (ASCT) AND THALIDOMIDE CONSOLIDATION, IS FEASIBLE IN OLDER PATIENTS WITH MULTIPLE MYELOMA AND CAN ACHIEVE A MYELOMA-FREE HARVEST

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Background. Bortezomib (BOR) and Cyclophosphamide (CY) have been recently associated in young patients with Multiple Myeloma (MM), achieving a high response rate, with good safety profile; moreover this schedule does not affect PBSC collection. Recently BOR has also been combined with High Dose Melphalan (HD-MEL), without affecting the engraftment and the morbidity after ASCT; Thalidomide (Thal) is active and well tolerated as consolidation after chemotherapy or ASCT. *Aims.* We started a pilot study in high risk MM elderly patients, fit for ASCT, combining BOR, CY and dexamethasone (DEX) as induction and mobilizing therapy (CY-BOR), followed by ASCT with supplemented BOR-HD-MEL. The main end point was the depth of response (percentage of CR) after the whole program; secondary end points were: percentage of clearance of Minimal Residual Disease (MRD) assessed by flow cytometry, both in the harvest and *in vivo* (in bone marrow before and after ASCT and after thalidomide), safety, PFS and OS. *Methods.* Patients received four 3 weeks courses of BOR 1.3 mg/m^2 and DEX 40 mg/day i.v. (days 1,4,8,11) and CY i.v. 300 mg/m^2 (days 1,8,15). Patients achieving at least PR, were mobilized after BOR and DEX standard dose (days 1,4,8,11) with CY 3 g/m^2 (day 8); GCS-F was started at day 9. Patients collecting an adequate PBSC amount underwent ASCT with HD-MEL (day -1) combined with BOR (1.3

mg/m^2 on days -6, -3, +1, +4). Three months after ASCT, patients not in CR or in CR but with evidence of MRD (by flow cytometry), received thalidomide 100 mg/day until progression or unacceptable toxicity. *Results.* 26 patients (median age: 66, range 52-77) were enrolled: 22 were evaluable for response before PBSC harvest: 18 responded to induction therapy (82%), 2 achieving PR (9%), 10 VGPR (46%), 4 nCR (18%), 2 CR (9%); 4 patients had resistant disease (18%). 17/22 patients were mobilized: in 14 we collected PBSC (median 5.5; range 2.5-11.2×10^6 CD34$^+$ cells/kg) and performed ASCT. Conditioning was well tolerated and followed by quick and complete engraftment, without major toxicities. With a minimum follow-up of 100 days after ASCT, all pts are alive, maintaining or improving the response: 5 VGPR, 2 CR, 7 nCR; 9 of them started the consolidation with thalidomide and only one relapsed. With a median follow up of 400 days (range: 74-753) all 26 pts are alive. We did not observe thromboembolic events; 3 pts experienced grade 3 neurotoxicity, requiring reduction of dose of BOR. MRD evaluation, by flow cytometry analysis, shows complete clearance of clonal plasmacells in 73% of PBSC harvests and in 29% of patients evaluated, before and after ASCT. *Conclusions.* These preliminary data show that this aggressive approach in fit elderly patients with MM is feasible, allowing to achieve a rapid and deep response, with a clonal plasmacells free harvest in the majority of cases (73%). Whether this will also translate in an *in vivo* MRD clearance, evaluated by Quantitative Real Time PCR with patients' specific probes (which is still ongoing), it has to be confirmed by a longer follow up.

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BORTEZOMIB - MAINTENANCE POST AUTOLOGOUS STEM CELL TRANSPLANTATION (ASCT) IMPROVES REMISSION DURATION AND QUALITY OF RESPONSE IN ADULT (< 65 Y) MULTIPLE MYELOMA (MM) PATIENTS: AN UPDATE

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Background. ASCT is the standard approach in adult MM patients (pts), however post ASCT disease progression may occur. *Aims.* In December 2007 we activated a single center study to assess the impact of Bortezomib maintenance a) on time to progression (TTP) and time to next treatment (TNT) b) the feasibility and toxicity. *Methods.* Between October 2002 and July 2008, at our Unit, 25 newly diagnosed MM pts (median age 59.5y, range 38-67) underwent single (9), or tandem (16) ASCT. Of these, 13 pts autotransplanted (8 single and 5 tandem) not receiving any treatment post-ASCT, were considered as control group (CG), while the remaining 12 transplanted from December 2007 to September 2008, received Bortezomib maintenance. (BG). Maintenance schedule consisted of Bortezomib as single agent given at dosage 1.5 mg every 15 days until progression. Response was evaluated according to the International Myeloma Working Group criteria, minimal residual disease (MRD) was assessed every 3 months on bone marrow (BM) samples by 6-colour BDFACS CANTO II. Abnormal plasma cells were identified as CD38, CD138, CD19, CD20, CD45, CD56, CD117, CD28, CD200 positive with a sensitivity level ≤ 1×10^{-5} (<0.01). Peripheral neuropathy (PN) was monitored before maintenance start, then every 3 mo by neurophysiologic motor and sensory conducting tests.

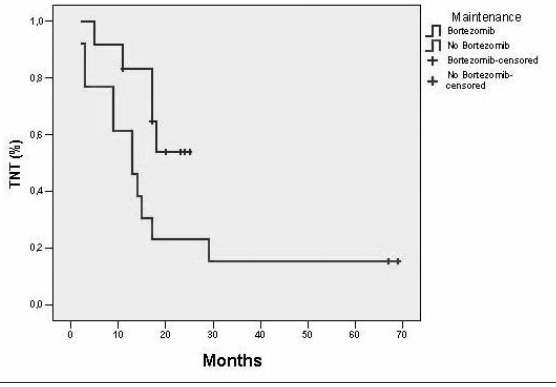

Figure. Time to next treatment.

Results. In the BG maintenance was started in a median time of 3.1 mo (range 1.7-13.7), post ASCT, 6 pts were in CR orVGPR, 4 PR, and 2 SD; the overall response rate was 83%, with 50% CR+VGPR. As of February 2010, after a median maintenance of 19.5 mo (range 4.1 -24), all 12 pts are alive. 6 (50%) in CR or VGPR (2 of these in sCR), 1 PR, 1 SD, 2 progressive disease and 2 pts who relapsed with an isolated extramedullary disease after 17 and 18 mos of maintenance, respectively. It is surprising that FISH analysis in 4/5 progressive-relapsed disease during maintenance had 13q- abnormality at diagnosis. Since not all pts underwent 2nd ASCT, TTP and TNT was evaluated from 1st ASCT. In the BG median TTP is 33 mo, whereas in the CG median TTP is 12 mo (P=0.01). Median TNT in BG has not yet been reached, whereas in the CG median TNT is 13 mo (P= 0.04). At median follow up of 40 mo (19-47) median OS has not yet been reached in BG, In CG at median follow up of 70 mo (31-96) median OS is 58 mo (P=0.023). Finally, none of pts in the BG experienced grade 3 or 4 haematologic toxicity and/or PN requiring dose reduction or drug discontinuation. *Summary and conclusions.* The preliminary results, even in a limited cohort of pts, suggest that Bortezomib as single agent in post-ASCT is active and safe to prolong TTP, TNT and able to improve response quality. However these preliminary results need to be confirmed by a longer follow-up and a randomized multicenter study.

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THALIDOMIDE DOES NOT AFFECT STEM CELL MOBILISATION IN PATIENTS WITH MULTIPLE MYELOMA

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Background. Over the past decade, thalidomide-containing regimes such as Cyclophosphamide, Thalidomide and Dexamethasone (CTD) have become first line therapy for patients fit enough for autologous stem cell transplantation. It is therefore essential that thalidomide does not have a detrimental effect on mobilisation of peripheral blood stem cells (PBSCs). A recent retrospective analysis (Auner *et al.* ASH abstract 2147, 2009) comparing CTD to regimes not containing thalidomide suggested a lower yield of CD34$^+$ PBSCs and a higher rate of mobilisation failures in the CTD group. *Aims.* Our aim was to see if introducing thalidomide-containing regimes such as CTD as first line chemotherapy has had an impact on the success of mobilisation of PBSCs in myeloma patients at our institution. *Methods.* We retrospectively analysed data on 121 consecutive patients with multiple myeloma who underwent PBSC harvest between 2004 and 2009. 73 patients treated with CTD were compared to 48 control patients receiving regimes not containing thalidomide: VAD (Vincristine, Adriamycin and Dexamethasone; n=35), ZDEX (Idarubicin and Dexamethasone; n=.10) or Velcade/Dexamethasone (n=3). The majority of patients were primed with cyclophosphamide 1.5 g/m^2 and either 5 or 10 mcg/kg GCSF but four patients received ESHAP (Etoposide, Methylprednisolone, Cytarabine and Cisplatin)chemotherapy and two patients received GCSF alone. Apheresis was performed when the peripheral blood human progenitor cell (HPC) count on a Sysmex SE2100 haematology analyser was > 0.045/μl or a peripheral blood CD34 count was >8×10^3 mL. If neither of these targets were reached, the patient was not apheresed. The harvest target was 4 - 6×10^6 CD34$^+$ cells/kg with a minimum of 2×10^6 CD34 + cells/kg. We compared the yield of CD34$^+$ stem cells, the number of apheresis days and the rate of failed mobilisation (no collection or yield <2×10^6 CD34$^+$ cells/kg) in the CTD patients and the control group. *Results.* Patients had received between 3 and 8 courses of CTD (5.2±1.5) and between 4 and 8 (5.2±0.98) of regimes without thalidomide. There was no significant difference between the two groups in the number of patients who failed to mobilise sufficient PBSCs (CTD 8.2±28% *vs.* controls 10±31%, P=0.35). The yield of CD34$^+$ cells/kg in the CTD patients was similar to the control group (CTD 6.73±3.84 *vs.* control 6.94±3.80, P=0.68). The mean duration of apheresis in the CTD arm was 1.64 ±0.69 *vs.* 1.70±0.64 days in the control arm (P=0.67). *Conclusions.* These results suggest that the use of CTD at induction in patients with myeloma has no adverse effect on PBSC mobilisation and so does not impact on the ability to proceed to autologous stem cell transplantation. Differences between our findings and those of other groups may relate to priming regimes used or patient factors such as prior radiotherapy.

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CIRCULATING LEVELS OF VISFATIN AND HIGH MOLECULAR WEIGHT ADIPONECTIN IN MULTIPLE MYELOMA: A CASE-CONTROL STUDY

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Background/Aim. Recent evidence suggests that obesity may be implicated in the etiology of multiple myeloma (MM). A novel hormone of the adipose tissue, visfatin (known as pre-B cell colony-enhancing factor) is a cytokine which is expressed in visceral fat exerting insulin mimicking effects and correlating with obesity. We thus attempted to explore whether altered secretion of the visfatin and high molecular weight (HMW) adiponectin, an adipokine linked with several obesity and insulin resistance associated malignancies including leukemia, may underlie this association. We have designed a case-control study to investigate the role of serum visfatin and HMW adiponectin in the etiopathogenesis of MM after adjusting for a potential confounding effect of age, gender, body mass index (BMI), and other adipokines. We also explored associations between visfatin and HMW adiponectin with prognostic factors of MM. *Methods.* Blood samples were collected from seventy three patients with incident, histologically confirmed MM and from an equal number of hospital controls admitted for non-neoplastic, non-infectious conditions and matched on gender, age (±5 years) and year/month of diagnosis (±1 month) between 2001 and 2009. Serum visfatin and HMW adiponectin concentrations were measured using ELISA (Phoenix Pharmaceuticals, Burlingame, CA, USA and ALPCO Diagnostics, Salem, USA respectively). MM prognostic parameters such as C-Reactive Protein (CRP), LDH, calcium, β-2 microglobulin (BMG) and erythrocyte sedimentation rate (ESR) were determined. Statistical analysis of the data was performed using univariate and multivariate analyses with SPSS version 17 for Windows. *Results.* Cases presented significantly higher body mass index (BMI) and elevated levels of serologic prognostic parameters of MM than controls. Cases had significantly lower serum levels of visfatin (mean±SD: 15.6 ng/mL±8.6) than controls (mean± SD: 28.7 ng/mL±15.9, P<0.001). Also, cases presented significantly lower serum levels of HMW adiponectin (mean±SD: 8.5 μg/mL±6.6) than controls (mean± SD: 14 μg/mL±9.6, P<0.001). Hypoadiponectinemia was associated with higher risk of MM after adjusting for age, gender, time at diagnosis, BMI and serum levels of other adipokines (P=0.002). Although, total and HMW adiponectin were both significantly inversely associated with MM when modeled either in quartiles or continuously, HMW didn't offer any substantial additional predictive value over total adiponectin (OR=0.92 *vs.* 0.91 for a 1 μg/mL change, respectively). Adjusting for the previous factors, lower levels of visfatin were found to be associated with significantly higher risk for MM (OR=0.89 and 95% Confidence Intervals: 0.85-0.94). No significantly different HMW adiponectin levels were found among different paraprotein classes and MM stages. In MM patients, serum visfatin was significantly positively associated with CRP, LDH, BMG, and ESR. Visfatin was significantly different among multiple myeloma stages with higher stages presenting higher visfatin levels (P<0.001). No significantly different visfatin levels were found among different paraprotein classes (P=0.65). *Conclusion.* HMW adiponectin may present a protective role in MM, whereas serum visfatin levels may be decreased via a compensatory response to the upregulation of other inflammatory parameters etiologically linked to MM such as IL-6. These observations need to be replicated and warrant further investigation.

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UNUSUAL EXTRAMEDULLARY PLASMACYTOMAS APPEARING DURING LONG-LASTING LENALIDOMIDE TREATMENT IN MULTIPLE MYELOMA: MUCH MORE THAN A CASUAL CLINICAL OBSERVATION?

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Background. The efficacy and safety of oral Lenalidomide plus High-Dose Dexamethasone (LD) in patients with relapsed-refractory MM was investigated in two Phase III large, randomized, multicenter, double-blind, placebo-controlled studies: the North American trial, MM-009 (n=354) and the European/Australian trial, MM-010 trial (n=351); [Dimopoulus *et al.*, Haematologica 2005; 80: 160]. As a result of MM-009 and MM-010 trials, LD reached an ORR of 61, 2- 58%, a CRR of 26, 5- 13,6% and a median TTP of 15 and 13,3 months, respectively. Recent publications has reported an increased risk of relapse as extramedullary plasmacytomas in MM treated with thalidomide containing regimens [E. Katodritoua *et al.*, Leukemia Research 2009; 33: 1137]. *Aims.* To review our recent clinical experience with the combination of LD in relapsing/ refractory MM and to describe the relapses after LD or the progressions during long-lasting continuous treatment with LD. *Patients and Methods.* We did a retrospective study from June 2007 to February 2010. We obtained efficacy and safety data by reviewing medical records from MM patients receiving treatment with LD and Data dispensing were collected from Landtools ® software.

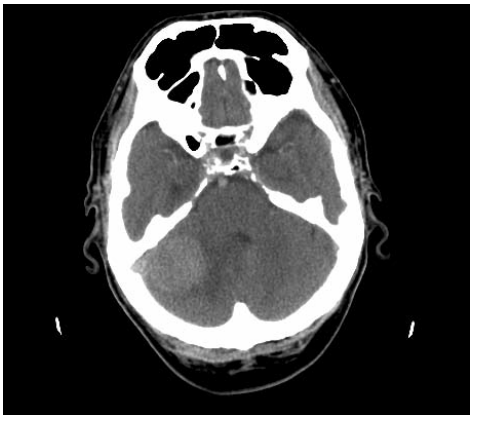

Figure.

Results. We identified 19 consecutive patients MM treated with LD (12 males and 7 females with a median age at the beginning of LD of 60 (22-82) years. Patients received a median of 5 (1- 21) cycles of LD. The median daily dose of Lenalidomide was 15 (5- 25) milligrams. Up to 15 patients were evaluable for response; 12 of 15 achieved an Objective Response (80%). There were 7 of 12 responding patients who did not exhibit progressive disease (1 submitted to autologous transplantation, 3 submitted to allogeneic transplantation, 3 showed a sensitive relapse after autologous transplantation). The other 5 Lenalidomide-responding patients displayed progressive disease, showing extramedullary plasmacytomas without evidence of bone marrow disease progression, as follows: 4 patients during the course of Lenalidomide-treatment exhibited extramedullary disease (a giant right pelvic plasmacytoma of 9×5 cms, a right iliac bone plasmacytoma, a neuromeningeal plasmocellular infiltration and a right mandibular plasmacytoma associated to an area of mandibular osteonecrosis due to zolendronate), diagnosed after 12, 11, 3 and 9 cycles respectively; the remaining patient suffered from a brain mass located in the right cerebellar hemisphere 14 months after receiving treatment with 6 cycles of LD, he underwent a tumoral biopsy that disclosed an extramedullary neoplasia of highly prolifferative plasmoblastic cells and die in the immediate post-surgical period. *Conclusions.* We have observed an unexpectedly high incidence (41%) of extramedullary disease among MM patients with sensitive to Lenalidomide-therapy disease. Lenalidomide combinations might offer a good control of the disease in relapsing/ refractory MM, but physicians should be alert of extramedullary relapses ("escape phenomenon") which confer a poor prognosis in MM.

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CD56 EXPRESSION AND TREATMENT OUTCOME WITH BORTEZOMIB THERAPY IN PATIENTS WITH RELAPSED PLASMA CELL MYELOMA

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Background. Approximately 67-79% of cases of plasma cell myeloma

aberrantly express CD56. It has been postulated that CD56 negative (CD56-) myeloma may develop from a less mature plasma cell and be associated with a worse outcome, though the evidence for this has been conflicting. Bortezomib is a peptide boronate inhibitor of the proteasome and is a highly effective therapy in relapsed and refractory myeloma. *in vitro* studies suggest that bortezomib therapy targets only $CD138^+CD56^+$ plasma cells.[1] *Aims.* We hypothesised that the presence or absence of CD56 may predict response to bortezomib therapy in the clinical setting in relapsed and refractory patients. *Methods.* We retrospectively analysed 25 patients (12 M: 13 F) with relapsed myeloma on whom diagnostic bone marrow immunophenotyping results were available. The median age of the cohort was 62 years (range 32-81). They had received a median of 2 previous lines of therapies, the most common being cyclophosphamide, dexamethasone and thalidomide (16/25). Four patients (4/25, 16%) has received a prior autograft. CD56 expression was assessed, prior to any therapy, by flow cytometry (FacsCantoII, Becton-Dickinson) utilising a CD138 live-gating strategy on bone marrow. A response to therapy was assessed according to published guidelines after 4 cycles of bortezomib. *Results.* Fifteen of 25 patients aberrantly expressed CD56. In the $CD56^+$ group, a partial response was seen in 11/15 (73%) patients. In the CD56- group, a partial response was seen in 4/10 (40%) of patients and a very good partial response in 1/10 (10%). Overall responses in the $CD56^+$ *vs.* $CD56^-$ group were 73% *vs.* 50% (Fisher's exact test, P=0.22). The median estimated survival in the $CD56^-$ group was 61 months; median survival was not reached in the $CD56^+$ group (log rank P=0.09). *Conclusion.* Though there appeared to be a trend towards higher responses in $CD56^+$ patients, who also appeared to have a trend towards better overall survival, however, given the relatively small sample size, neither reached statistical significance. These interesting observations suggest a need for further studies to assess the impact of CD56 status on patients treated with bortezomib, and these may elucidate whether CD56 expression could influence the decision of treating relapsed patients with bortezomib or other novel agents.

Reference

1. Kirshner et al. Blood 2008;112:2935-45.

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THE MAINTENANCE THERAPY WITH THALIDOMIDE IN ELDERLY PATIENTS WITH MULTIPLE MYELOMA

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Background. Current treatment approach to the newly diagnosed patients (pts) with multiple myeloma (MM) is based on two elements: age and risk factors. Main goals of treatment of elderly patients are prolongation of survival accompanied with maintenance of good performance and hospitalization independence. The aim of study was to analyse results of the thalidomide maintenance in elderly (>65yrs) MM pts. *Patients and methods.* The study included 80 newly diagnosed MM pts (45 male/35 female, mean age 68 yrs, range 66-83yrs). IgG myeloma was diagnosed in 47pts, IgA in 17pts, light chains in 15pts and non-secretory in 1pts. According to the clinical stage (CS, Durie-Salmon), patients were distributed as follows: II 37pts; III 43pts. Regarding ISS score, the group included: ISS1 23pts; ISS2 22pts; ISS3 35pts. Renal impairment was present in 15pts. The study included 3 arms of induction treatment according to the regimens: a) MP (30/80pts, 37,5%); b) MPT (35/80, 43,7%); and c) Thal-Dex (15/80, 18,7%). Maintenance therapy with Alfa-Interferon (aIFN) was applied in 30pts treated with MP chemotherapy. Thalidomide maintenance (100 mg/day) was applied in 50/80pts (62,5%) treated with MPT/Thal-Dex induction with median duration 16m (range 4-28m). Routine thromboprophylaxis was applied in all pts receiving thalidomide. *Results.* In the group of pts treated with MP, PR (IMWG criteria) was achieved in 12/30pts (40%). The overall response rate were significantly higher (P<0,001) in pts treated with MPT (CR/VGPR/PR: 27/35pts; 77,3%) and Thal-Dex (CR/VGPR/PR: 10/15pts; 66,7%). Median follow-up was 34 m (18-60 m). The 3-yrs probability of event-free and progression-free survival was significantly improved in the group treated with MPT/Thal-Dex induction and thalidomide maintenance (MP+aIFN: EFS 20%, PFS 25% *vs.* MPT+Thal: EFS 40%, PFS 54% *vs.* Thal-Dex +Thal: EFS 46%, PFS 50%, P< 0,003). Furthermore, according to the analysis of 3-yrs probability, pts treated with thalidomide maintenance had a significantly longer overall survival (MP+aIFN: 29% *vs.* MPT+Thal: 48% *vs.* Thal-Dex +Thal: 51%, P< 0,0025). The main reason for thalidomide discontinuation was peripheral neuropathy recorded in 37/50pts (74%) with occurrence of grade 3-4 toxicity in 6/37pts. Thrombosis was recorded in 4/50 treated with thalidomide maintenance. *Conclusion.* Application of the thalidomide maintenance resulted with improvement in the quality of response, its duration and overall survival of the elderly myeloma patients. Still, limitations of thalidomide maintenance mainly due to the toxic effects and possibility to induce more resistant relapse, indicates further studies of such therapy in terms of recommended duration and dosage.

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SERUM FREE LIGHT CHAINS ASSAY IN DIAGNOSIS OF MONOCLONAL PLASMA CELL DISORDERS - SINGLE CENTER EXPERIENCE

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Serum Free Light Chains are important markers for diagnosis and monitoring patients with monoclonal plasma cell disorders (PCDs). Automated immunoassay that measures serum free light chains (FLCs) has been recently introduced in our lab and this report present our experience. *Aims.* to evaluate the utility and efficiency of FLCs along with immunofixation electrophoresis (IFE) and serum protein electrophoresis (SPEP) in diagnosis of patients with monoclonal plasma cell disorders and for monitoring response to treatment. *Methods.* We analyzed samples from 182 patients with diagnosis or suspicious of PCDs. The FLC assay (FREELITETM; Binding Site) was performed on a Dade Behring, BN ProSpec automated nephelometer. The FLC κ/λ ratio was used to determine a positive or negative test and data was analyzed based on previously established normal ranges. A κ/λ ratio less than 0.26 (positive for λ FLC) or greater than 1.65 (positive for κ FLC) were considered positive. In the same time, for each patient were performed SPEP and IFE at the patient's initial presentation and during follow- up. *Results.* In this study we identified: 122 patients with intact immunoglobulin multiple myeloma (IIMM); 13 patients with light chain multiple myeloma(LCMM); 2 patients with nonsecretory multiple myeloma (NSMM); 2 patients with smoldering multiple myeloma (SMM); 8 patients with monoclonal gammopathies of undetermined significance (MGUS), 6 patients with solitary plasmocytoma(SP), 4 patients with lymphoplasmcytic lymphoma (LPL), 22 patients with primary systemic amyloidosis (AL), and 3 patients with Waldenstrom disease (WD). The monoclonal protein were similarly identified by FLCs assay, IFE and SPE in all patients with IIMM, SMM, MGUS, WD. For patients with LCMM classical IFE and SPE failed to demonstrate monoclonal protein in 11 cases. FLC assay help us to identified the monoclonal light chain in all of these cases. Among the 22 AL patients at the initial presentation 16 of them showed high levels of κ or λ light chains, but IFE were positive only for 8 patients. The serum FLC κ/ λ ratio was abnormal in 90.9% of patients with IIMM, 84.6% of patients with LCMM, 50% of patients with NSMM, 62.5% of patients with MGUS, 66.6% of patients with SP, 75% of patients with LPL, 100% of patients with WD and 72.7% of patients with AL. The most important fact of using FLC assay in our patients with PCDs is the clinical relevance of early FLC "response"after therapy or "relapse"in patients with measurable serum immunoglobulin free light chains, specifically in IIMM, AL and MGUS. *Conclusions.* The FLC assay is complementary to IFE and SPE in detecting the monoclonal protein in patients with PCDs, but, in addition, the sFLC assay has shown significant clinical application for monitoring response to therapy. FLCs assay has proven to be more sensitive than IFE in the diagnosis of patients with LCMM and AL.

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THE IMPACT OF FRONTLINE TREATMENT ON SURVIVAL OUTCOMES IN MULTIPLE MYELOMA (RETROSPECTIVE STUDY)

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Background. Multiple myeloma (MM) is a plasma cell malignancy characterized by an indolent clinical course with recurrent remission and relapses. The significance of attaining a complete remission (CR) rather than a partial response after frontline treatment, and its impact on overall survival (OS) remains controversial. *Aims.* We evaluate the effectiveness of the various frontline regimens used and whether best responses

to frontline therapy are good surrogates for OS. *Methods.* Previously untreated MM patients diagnosed from 2000-2007 with complete clinical data were included. Criteria for partial response (PR) required reduction of serum myeloma protein level by >50%, while a complete remission (CR) was defined by negative findings on immunofixation. OS curves were estimated by the Kaplan-Meier method. *Results.* 110 patients with complete treatment data were available for review. The median age was 62 years. 22 (20%), 31 (28.1%) and 47 (42.7%) patients presented with ISS stage I, II and III disease respectively. The induction therapy received was alkylating agents - 15 (13.7%), VAD based - 42 (40.9%), Melphalan Based - 23 (20.9%) and Bortezomib Based therapy - 27 (24.5 %) respectively. 33(30%) and 67 (60.9%) patients attained CR and PR respectively after frontline treatment. The median progression free survival was 2,8 years and independent of response status. Patients who attained CR however, had a significantly better median OS at 6,7 years compared to those who achieved PR at 4,3 years. No significant differences were observed in the response rate among the different frontline regimes, although bortezomib-based induction showed a trend towards more rapid reduction of M-band. *Conclusions.* Achievement of CR seems to confer a more favorable survival. Survival analysis is important for better understanding the contributions of treatment, particularly bortezomib and transplant, as well as other factors in these patients.

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VALUE OF MAGNETIC RESONANCE (MR) AND POSITRON EMISSION TOMOGRAPHY (PET) FOR DIAGNOSIS AND FOLLOW UP OF BONE LESIONS IN MULTIPLE MYELOMA (MM)

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Up to 90% of symtomatic MM patients develop osteolytic lesions during the course of the disease. The lesions rarely heal even when patients achieve complete remission (CR). Conventional radiography (X-rays), computed tomography (CT) and MR are all used to diagnose the extent of bone disease but they are limited for the follow-up of the disease. During last decade, PET-CT has proved to be useful for staging and monitoring response to treatment in solid tumours and lymphomas. Moreover, five MR imaging patterns of marrow involvement in MM have been described and they can be related with tumor burden. The aim of this study was to analyze the ability of PET-CT and MR to evaluate bone disease in MM at diagnosis and during the course of the disease. In addition to biological and clinical assessment, each patient underwent X-rays, spine and pelvis MR and PET-CT studies. We studied a total of 17 patients in different moments: 10 at the moment of diagnosis MM, and 11 when CR was reached (so 4 of them could be studied before and after treatment). From the group 10 patients studied at the moment of diagnosis of symtomatic MM: there was concordance between PET-CT and X-rays in 5 patients, and there was discordance in the other 5 patients (2 PET-CT positive but X-rays negative, 2 X-rays positive and PET-CT negative, and 1 patient positive in both studies, but affecting different regions). The marrow patterns by MR were abnormal in all the 10 patients. In five of the 10 patients a new evaluation was performed after achieving any treatment response (4 CR and 1 partial response); while the PET-CT scan was normal in all cases after the therapy, the pattern of marrow involvement by MR remained pathological. Finally, we studied another group of patients that were in CR (negative immunofixation) after treatment (n=11). Four cases showed a normal marrow pattern by MR and PET-CT was negative in 9 cases. Conclusion. The pattern of marrow involvement seen with MR in symptomatic MM patients, can return to normal in patients achieving CR; our study suggests that the normal pattern of medullar signal by MR would occur with some delay after reaching CR. PET-CT needs of other radiological techniques for the correct assessment of bone disease in patients with MM at the time of diagnosis, but is able to detect resolution of lesions when CR is obtein. Nevertheless, the value of PET-CT + in patients with complete response (IF-) needs further evaluation.

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PREDICTIVE VALUE FOR SURVIVAL OF POST-TRANSPLANT DAY+14 BONE MARROW PLASMA CELL PERCENT IN MULTIPLE MYELOMA PATIENTS WHO UNDERGONE SINGLE AUTOLOGOUS TRANSPLANTATION

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Backgrounds. Autologous stem cell transplantation (ASCT) has become the treatment of choice for eligible patients with multiple myeloma (MM).In recent studies, pre- and post-transplant response after ASCT were reported to predict the outcome in MM patients. *Aims.* We analyzed retrospectively the prognostic influence of post-transplant day+14 bone marrow plasma cell percent (BMPCp) and other pre-transplant characteristics on progression-free survival (PFS) and overall survival (OS) in 39 MM patients received ASCT between 2003 and 2008. *Methods.* All patients received induction chemotherapy with vincristine, adriamycin and dexamethasone (VAD) and ASCT after high dose melphalan alone conditioning therapy. We evaluated the influence of post-transplant day+14 BMPCp (≥2% *vs.* <2%), ISS stage (II *vs.* III), response after 3 cycle of VAD therapy (complete response (CR) *vs.* non-CR), deletion of chromosome 13q (del(13q)) (presence of the abnormality *vs.* absence) and BMPCp at diagnosis (≥50% *vs.* <50%) on PFS and OS. *Results.* Median follow-up duration was 28.0 months. International staging system (ISS) at diagnosis were stage II (n=25) and stage III (n=14). The type of M protein were IgG (n=23), IgA (n=12) and others (n=4). Median BMPCp at diagnosis was 43.0 % (range, 11-57%) and chromosomal abnormalities at conventional cytogenetic study were found in 15 patients. Deletion of chromosome 13 by FISH was found in 8 patients. After induction VAD therapy, 12 patients achieved complete response (CR). Infused mean CD 34^+ stem cell dose was 4.1×10^6/kg (range, $2.1\text{-}6.1\times10^6$/kg). Median BMPCp at post-transplant day+14 was 0.7% (ran! ge, 0-4.0%). Analysis of different cut-off levels between the 25% and 75% quartile (0.2-2.2%) using the log-rank test determined that BMPCP=2% as the cut-off point yielded the highest difference in PFS and OS, which was used as cut-off level in statistical analysis. Comparing to baseline characteristics between the two groups (BMPCp ≥2% *vs.* <2%). In univariate analysis, the five prognostic factors (post-transplant BMPCp, del(13q), CR after 3 cycles after VAD therapy, ISS fatge III and BMPCp at diagnosis) were significantly correlated with PFS and OS. Multivariate analysis revealed that post-transplant day+14 BMPCp ≥ 2% (PFS, HR=4.426, P=0.008; OS, HR=3.545, P=0.038) and CR after 3 cycle of VAD therapy (PFS, HR=0.072, P=0.014; OS, HR=0.055, P=0.015) were independent prognostic parameters on RFS and OS compared with other characteristics. *Conclusions.* It appears that post-transplant day+14 BMPCp would be an important and useful parameter predicting the outcome for the patients with MM received ASCT.

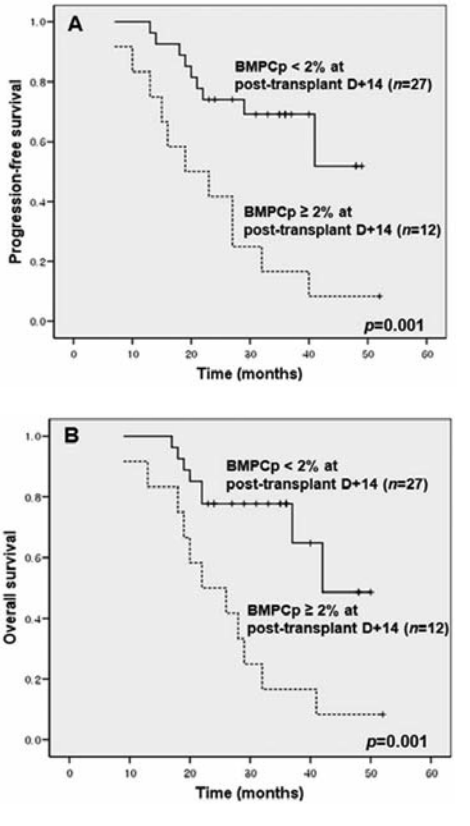

Figure.

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VASCULAR ENDOTHELIAL GROWTH FACTOR AS PROGNOSTIC FACTOR OF CLINICAL COURSE OF MULTIPLE MYELOMA

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Background. Despite generally low overall survival of patients with multiple myeloma (MM) certain group of patients live more than 10 years even being treated with conventional therapy. This fact can be explained by considerable prognostic heterogeneity of multiple myeloma. Introduction of the new highly effective therapeutic methods significantly improves treatment outcomes irrespective of prognostic factors. Therefore the issue of searching for the new and independent prognostic factors for multiple myeloma is currently important. *Aims and methods.* Serum levels of β-2 microglobulin and vascular endothelial growth factor (VEGF) were studied in 31 patients with MM during the course of treatment. Serum levels of VEGF and β-2 microglobulin were determined by immune-enzyme analysis using standard laboratory kits "Human VEGF"(BIOSOURCE, USA) and "β-2 microglobulin"(DAI, USA). According to the results obtained, all the patients were divided into 2 groups: group 1 - with lower (< 5,5 mg/L) and group 2 - with higher (>5,5 mg/L) levels of β-2 microglobulin. Patients of both groups were evaluated for treatment response, according to which each group was further subdivided into 2 subgroups - first one included non-responders (NR) and those who achieved any response (complete, partial or minimal response) were assigned to the second subgroup. Levels of β-2 microglobulin and VEGF were determined during the course of treatment. *Results.* In the first group of patients with low baseline values of β-2 microglobulin its level was 2,37 mg/L in 14 responding subjects and 1,64 mg/L in 6 non-responders. In the course of treatment this parameter tended to decrease irrespective of treatment efficacy. However, this decrease was much more significant in patients of the second subgroup who achieved response, whereas in non-responders the level of β-2 microglobulin was found to reduce only by half. As for VEGF, its baseline levels in patients of the first group were higher in responders compared to non-responding subjects and equaled 280,87 pg/mL and 205,80 pg/mL respectively. During the course of disease serum levels of this cytokine became almost normal in patients with the positive treatment results, at the same time non-responding patients were found to show increase of serum VEGF. The levels of VEGF at baseline in patients of the second group with high β-2 microglobulin values (high risk group) differed between the two subgroups depending on the treatment efficacy and were 196,70 pg/mL and 341,18 pg/mL in responding subgroup and non-responders respectively. After anti-myeloma therapy this parameter showed certain elevation in responders whereas in non-responding subgroup of patients, although decreasing, it remained two times higher than VEGF levels of healthy volunteers. This tendency may be considered as predicting factor of disease progression in this group of patients. Conclusion. The results of this study suggest that combined evaluation of serum levels of β-2 microglobulin and VEGF at baseline and at different time points during the anti-myeloma treatment may serve as prognostic criteria in patients with multiple myeloma.

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BENEFITS OF NOVEL AGENTS ON MOBILIZATION AND ENGRAFTMENT IN AUTOLOGOUS STEM CELL TRANSPLANTATION FOR MULTIPLE MYELOMA PATIENTS

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Background. The introduction of novel agents (NA), such as proteasome inhibitors and IMIDs, in recent years has improved the outcomes for multiple myeloma (MM) patients. Autologous stem cell transplantation (ASCT) is actually recommended as initial therapy in younger patients but there are contradictory data about influence of NA on mobilization as well as time of engraftment. *Aims.* To evaluate the influence of the NA on mobilization and time to the engraftment in consecutively treated MM patients who underwent ASCT in University Hospital La Paz, Madrid. *Methods.* We searched in our computerized database and reviewed the medical records of MM patients who underwent ASCT between 2003 and 2008 in our centre. Patients were divided in two groups: those who received NA as induction therapy and those who received conventional drugs (CD). Those patients who received lenalidomide as induction therapy were excluded for the analysis. Data were analyzed with the software SPSS 15.5 for Windows. *Results.* We have included 41 patients (18 men). Mean age was 59 y/o (r: 41-65) with the following type of MM: IgG / IgA / light chains / IgD / non-secretory: 20%, 13%, 6%, 1%, and 1% respectively. Induction regimen used was VAD in 10 patients, VBCMP/VBAD in 4, melphalan alone or melphalan + dexametasone in 5, cyclophosphamide + dexametasone in 6, bortezomib + dexametasone in 6, thalidomide, bortezomib, dexametasone in 6, and thalidomide + dexametasone in 4 patients. Twenty-three patients received autologous transplant as first line of treatment; 12 in 2nd line, 6 in 3rd line. Ten patients in 1st line received NA and 12 CD. Five patients in 2nd line received NA and 8 CD. Twelve patients required one apheresis (9 NA, 3 CD); 27, two apheresis (5 NA, 22 CD) and 2 patients needed three apheresis. In one patient treated with CD mobilization with cyclophosphamide was necessary. The median number of CD34×10^6/kg obtained was 5,3 (r: 3,5-6,3) for the NA group and 3,3 (r: 2,7- 4,9) for the CD group (p 0,036). The median of transfused red cells units was 2 (r: 0-3) in the NA group and 4 (r:2-6) in the CD group (P≤0,05). The median of platelets pool transfused was 2 (r: 2-4) and 6 (r: 3-7) respectively. The median of days with thrombocytopenia was 10 and 14 days and of neutropenia was 12 and 14.5 days in both groups respectively (P≤0.002). In the multivariable analysis, the NA had a direct effect over the reduction in the aplasia time post-transplant in 2 days (P:0.025, IC95%: 3.7-0.25) regardless the number of CD34 cells infused. *Conclusions.* In our series, the NA used for the MM induction treatment, excluding lenalidomide, are associated with an improvement in stem cell mobilization, as well as a reduction in the transfusion requirements and the engraftment after transplant.

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POLYMORPHISMS OF DNA REPAIR GENES IN MULTIPLE MYELOMA; NO ASSOCIATION WITH XRCC1(399) POLYMORPHISM, BUT THE XRCC4 (VNTR IN INTRON 3 AND 1394) AND XPD (751) POLYMORPHISMS IN ASSOCIATION WITH THE DISEASE IN TURKISH PATIENTS

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Background. Genetic polymorphisms of DNA repair genes seem to determine the DNA repair capacity, which in turn may affect the risk of Multiple myeloma (MM). *Aim.* This study aims to investigate the association between the polymorphisms in DNA repair genes (XPD, XRCC1, XRCC4) and clinical parameters in patients with MM, their effects on prognosis and their roles in susceptibility to MM. *Methods.* Some 60 patients, diagnosed with MM in the Section of Hematology at the Department of Internal Medicine in the Faculty of Medicine at Gaziantep University, and some 70 individuals as the healthy control group were included in the study. DNA isolation was performed using 2 cc of blood sample in tubes with EDTA for the control group and using bone marrow smear preparations for the patient group. Gene polymorphisms were detected with the PCR and/or PCR-RFLP method. The obtained results were compared both among each other and with clinical parameters. *Results.*When the genotype frequencies of XPD (751) and XRCC1 (399) genes were examined in terms of patient and control groups, no significant difference was detected, while a significant association was detected in XRCC4 (VNTR in intron 3 and 1394) polymorphisms in terms of genotype frequency. In terms of XPD (751) gene polymorphism, a significant association was found in MM patient group in terms of AA genotype and event-free survival (EFS) (P=0.047). When XRCC4 VNTR intron 3 polymorphism was compared in terms of genotype frequency, DD genotype was determined to be significantly low (P=0.012) in the MM patient group, whereas GG and TT genotypes were detected to be significantly low in the patient group in terms of the genotype frequency of XRCC4 (1394) polymorphism when compared to the healthy control group (P=0.015, P=0.010, respectively). *Conclusions.* These data provide support for the hypothesis that common variation in the genes encoding XRCC4 DNA repair proteins contributes to susceptibility to myeloma. These findings require further validation in independent populations.

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ASSOCIATION OF SERUM LEPTIN AND RESISTIN LEVELS WITH MULTIPLE MYELOMA RISK

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Background/Aim. The adipose tissue is currently considered an active endocrine organ participating in the modulation of insulin sensitivity and resistance, energy homeostasis, bone metabolism, inflammation, hematopoiesis, immunity and angiogenesis. Accumulating evidence supports a role for obesity in the etiology of multiple myeloma (MM). The distinct possibility exists that obesity may be linked to MM through altered adipokine secretion. Whether circulating leptin or leptin produced by bone marrow adipocytes and stromal cells, and its receptor OB-R may function as a growth factor/receptor-ligand system in malignant plasma cells acting in a paracrine fashion remains to be demonstrated. In this case-control study we investigated the role of serum leptin and resistin in the etiopathogenesis of MM and we explored their association with several established prognostic factors. *Methods.* Between 2001 and 2009, blood samples were collected from seventy three patients with incident, histologically confirmed MM and from an equal number of hospital controls admitted for non-neoplastic and non-infectious conditions and matched on gender, age (±5 years) and date of diagnosis with cases. Serum leptin and resistin concentrations were measured using ELISA (Avibion Human Elisa, Orgenium Laboratories, Helsinki, Finland and Phoenix Pharmaceuticals, Inc, Burlingame, CA 94010, USA, respectively). MM prognostic parameters such as C-Reactive Protein (CRP), LDH, calcium, β-2 microglobulin and erythrocyte sedimentation rate were determined. Statistical analysis of the data was performed using univariate and multivariate analyses with SPSS version 17 for Windows. *Results.* Patients had significantly higher height, weight and body mass index (BMI) as well as higher levels of serologic prognostic parameters of MM than control subjects. In univariate analysis, cases presented significantly lower serum levels of resistin (mean±standard deviation: 9.4 ng/mL±5) than controls (mean±standard deviation: 15.9 ng/mL±6.8, P<0.001). Also, patients presented significantly higher serum levels of leptin (mean±standard deviation: 27.5 ng/mL±17.6) than hospital controls (mean±standard deviation: 21.9 ng/mL±9.5, P=0.02). Adjusting for age, gender, date of diagnosis, BMI and serum levels of adipokines, higher levels of resistin were found to be associated with significantly lower risk for MM (P<0.001). In contrast to resistin, leptin showed no significant association with risk for multiple myeloma after adjustment with the previous variables (P=0.18). Amid cases, only leptin tended to be positively associated with CRP, LDH and calcium, and negatively with adiponectin. Furthermore, serum leptin was significantly different among multiple myeloma stages with higher stages presenting increased leptin levels (P=0.01). No significantly different leptin or resistin levels were found among different paraprotein classes. *Conclusion.* The results of our study don't provide support that leptin, a reflection of the degree of obesity, is independently associated with risk for multiple myeloma. The decreased resistin levels in MM patients could be associated with the upregulation of other adipokines or cytokines involved in MM. Further studies are needed to confirm these associations and to explore the mechanisms underlying leptin and resistin implications in MM and plasma cell dyscrasias.

1469

BORTEZOMIB TREATMENT AT HOME DOES NOT DECREASE EFFICACY, TOLERABILITY AND COMPLIANCE COMPARED WITH IN-HOSPITAL ADMINISTRATION

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Background. In Belgium in 2008 the MyCare@home® project was started, making it possible to treat multiple myeloma (MM) patients receiving bortezomib at home. Patients generally receive the first cycle and the first injection of subsequent cycles at the hospital. Trained nurses provide the administration at home after assessing the patient's health status and possible adverse reactions and consulting with the hematologist. To date, the service has been used for 117 patients in 23 centers, with no major infusion related reactions reported. eVOBS is an observational study in 7 countries designed to assess bortezomib treatment in daily practice. Some of the MyCare® patients were enrolled in eVOBS, allowing for subgroup analysis. *Aim.* Primary objectives were efficacy and tolerability. Secondary objectives included treatment and dose schedule, use of co-medications, and (in Belgium), quality of life (QoL). Aim of this analysis is to describe these parameters in the homecare population, and compare with a matched group. *Methods.* Adults were eligible if treated with bortezomib within approved indication. All bortezomib dosages and co-medications were permitted, except investigational therapies. Due to the non-interventional nature of the study, no predefined response criteria were mandated; these could include M-protein, EBMT, SWOG, or other. All patients signed informed consent. QoL assessment was conducted using the EORTC QLQ-C30, administered at the start of each treatment cycle. For comparison of safety and efficacy results, the homecare patients were matched with 17 hospital treated patients for age, line of treatment, disease stage and presence of pre-existing neuropathy. Because very few patients in the matched group completed the QoL questionnaires, for QoL results only homecare patients were compared with all hospital patients (N=138). *Results.* 155 Belgian patients were enrolled in eVOBS, of whom 17 received treatment at home. Reasons for the physician to choose home care were: patient mobility and transport difficulties, patient comfort and overburdened day clinic. Table shows baseline parameters and efficacy *Results.* In the matched group AEs were most frequent reason for discontinuation of treatment (53% *vs.* 20% in home group). Most frequent AEs - presented as all grades (gr.3-4 if present), home *vs.* matched - were: new onset neuropathy 57% (14% gr.3) *vs.* 58% (33% gr.3), fatigue 12 *vs.* 47%, nausea 24 *vs.* 24%, thrombocytopenia 18 *vs.* 24% (6% gr.3-4). On all subscales of the EORTC QLQ-C30, home patients had scores similar or slightly better than the total hospital group. *Conclusion.* Although the number of patients is limited, these results suggest that bortezomib treatment at home, in a controlled setting, is of at least comparable efficacy and tolerability as a treatment completed in the hospital. Aside from the potential benefit to the patient, this could also result in reduced costs.

Table. Baseline parameters and efficacy results.

Baseline parameters/ results	Homecare patients (N=17)	Matched group (N=17)
Age (years)	69.4	70
Time since diagnosis (yrs)	3.5	1.5
≥3rd line therapy	30%	30%
Median cycles given	5	5
ORR/CR/nCR (%)	59/18/6	59/6/6
PFS (months)	10.7	7.1

1470

SMALL BUT SIGNIFICANT OVERLAP IN SERUM FREE LIGHT CHAIN PARAMETERS IN INDIAN NON - MYELOMA AND MYELOMA SUBJECTS

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Background. Serum free light chain (sFLC) analysis for detection and monitoring of monoclonal gammopathies has been validated by several studies but none from India. We undertook this study to determine if sFLC parameters could clearly distinguish between myeloma patients and non-myeloma subjects in India who might be expected to be having higher sFLC on account of greater incidence of sub-clinical infec-

tions. *Methods.* Serum samples from 135 healthy subjects, 50 patients with active pulmonary tuberculosis , 25 with autoimmune disorders, 25 cases of CRF(chronic renal failure) and 51 myeloma patients were analyzed using serum protein electrophoresis , immunofixation when indicated and estimation of sFLC , the latter using FREELITE reagent kit and Hitachi autoanalyzer. Parameters analyzed were κ and λ levels, κ/λ ratios. *Results.* Median age of healthy subjects (115 males, 20 females) was 39.5 years (range 19-58), tuberculosis patients (50 males) 48 years (range 21-75), patients with auto immune disorders (13 males, 12 females) 36.5 years (range 17-60 years), patients with CRF (20 males, 5 females) 50 years (range 19-85 years) and myeloma patients (37 males, 14 females) 55 years (range 27-79 years). Serum k levels ranged from 6.8 - 70.7 mg/L in healthy donors, 19. 6 - 65.0 mg/L in tuberculosis, 8.9 - 68.3 mg/L in autoimmune disorders, 11.7-77.7 mg/L in CRF and 5.9-5420.0 mg/L in myeloma patients. Serum λ levels ranged from 3.3-66.7 mg/L in healthy subjects, 14.9-93.5 mg/L in tuberculosis, 4.3-90.8 mg/L in autoimmune disorders patients, 14.9 - 125.3 in CRF and 5. 4 - 7,700.0 mg/L in myeloma. The κ/λ ratio was 0.6-3.0 in healthy donors, 0.5-2.9 in tuberculosis, 0.4-1.1 in autoimmune disorders, 0.6-6.9 in CRF and 0.002-669.1 in myeloma patients. Ten of the 51 (19.6%) myeloma patients had low κ/λ ratio that overlapped with the non-myeloma subjects,especially the CRF cases. *Conclusions.* Indian individuals not having myeloma have higher κ and λ values and also higher κ/λ ratio than reported, possibly because of greater incidence of subclinical infections, a conclusion supported by the findings in tuberculosis patients. As the higher values of κ/λ ratio in non-myeloma subjects overlap with a small but significant number of myeloma patients, the distinction between the two groups, especially in Indian subjects, must often require correlation of multiple parameters. Though the κ/λ ratio is reported to be very sensitive for myeloma detection, study of a larger cohort of patients and healthy Indian subjects is needed to validate our findings.

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A MULTICENTER, OPEN LABEL STUDY OF VELCADE, MELPHALAN AND PREDNISONE IN RELAPSED/REFRACTORY MULTIPLE MYELOMA PATIENTS

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Background. Velcade has shown significant anti-myeloma activity in clinical studies. In newly diagnosed elderly patients (pts) the addition of Velcade to the standard oral Melphalan and Prednisone (MP) significantly increases response rate and event free-survival compared to MP. In this multicenter open label phase I/II trial we assessed dosing, efficacy and safety of MP plus Velcade (MPV) as salvage treatment for multiple myeloma (MM) pts. *Patients and Methods.* In order to assess the maximum tolerated dose (MTD), 19 MM pts in relapse or refractory, after one or two lines of treatment, including high-dose chemotherapy with stem cell support, conventional chemotherapy, Thalidomide, Velcade and Melphalan-based regimens, entered the first phase of the study. They were treated with Melphalan 24 mg/28 days (2 mg on Monday, Wednesday and Friday every week), Velcade 1.3 mg/m^2 as a bolus intravenous (IV) injection on days 1, 8, 15, 22 and Prednisone at the dose of 50 mg every other day, for a total of 9 cycles. The MTD will be evaluated after the first 3 VMP cycles. The MTD dose was defined by two parameters: the number of responses (at least 5 partial responses: PR) and the number (<10) of pts presenting the following toxicities: grade 4 neutropenia >4 weeks or grade 4 hematological toxicity except neutropenia or any grade 3 non-hematological toxicity. At the end of the first phase, a further series of 23 pts was enrolled. All (total=42) pts received acyclovir as prophylaxis. *Results.* Between March 2008 and March 2009, 19 pts were treated and 12 (60%) achieved at least a PR; 7 presented the following toxicities: 3 pts grade 4 thrombocytopenia and 6 pts grade 3 non-hematological toxicity (4 infections, 1 peripheral edema and 1 diarrhea). Based on these results and according to the study design, between March 2009 and January 2010 23 further pts were treated with the same schedule. At the time of writing, 10 pts have completed the study and 19 are ongoing, while 13 have dropped out of the study (11 due to progression of disease, PD, 1 consent withdrawal and 1 SAE). 31/42 pts received at least 2 cycles and were considered for response. 17/31 (55%) obtained at least a PR: 6 pts reached a near complete remission (nCR), 1 achieved a very good partial response (VGPR), 10 showed a PR. In addition, 2 pts showed a minimal response, 1 stable disease and 11 PD. Toxicity was observed, more frequently during the early cycles, in 12/42 pts (28%) and it was manageable. Major grade 4 hematological toxicities consisted of thrombocytopenia (4 pts). Major grade 3-4 non-hematological toxicities were infections (5), diarrhea (2), neuropathy (1), hepatic toxicity (1), peripheral edema (1). *Conclusions.* The weekly infusion of Velcade + MP induced a high proportion of responses and was well tolerated in relapsed/refractory MM pts. An update of these data will be presented at the meeting.

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PERIPHERAL NEUROPATHY CLINICAL COURSE DURING LENALIDOMIDE THERAPY FOR RELAPSED/REFRACTORY MULTIPLE MYELOMA: A SINGLE-CENTRE PROSPECTIVE NON INTERVENTIONAL STUDY

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In recent years, proteasome inhibitors and immunomodulatory drugs (IMiDs) have provided invaluable advantage in the therapy of multiple myeloma (MM) in terms of disease free survival and, to some extent, overall survival. However the benefits have sometimes been blunted by the occurrence of adverse effects which strongly impact patients'quality of life. Among them, chemotherapy-induced peripheral neuropathy (CIPN) represents one of the most disabling and often dose-limiting side effect. Lenalidomide, a second generation IMiD and structural analogue of thalidomide, has been claimed to have low toxicity on peripheral nervous system. In a single-centre prospective study we evaluated the natural history of CIPN in consecutive patients previously treated with bortezomib and/or thalidomide, who shifted to lenalidomide for relapsed or refractory MM. The aim was to evaluate the clinical course of CIPN during lenalidomide therapy. *Patients and methods.* 34 consecutive patients previously treated with bortezomib and/or thalidomide were treated with lenalidomide (25 mg daily for 21 day cycle) alone or associated with low dose dexametasone for relapsed/refractory MM. Patients previously treated with bortezomib and/or thalidomide, who developed a CIPN and whose MM was still in remission, were included as control. Neurological evaluation was planned at baseline, after 6 and 12 months from the beginning of lenalidomide therapy. Patients were assessed with the Total Neuropathy Score clinical version (TNSc); pain was assessed with the Numeric Rating Scale (NRS) Nerve conduction studies were performed at the beginning of the treatment and regularly during follow up. Lack of chemotherapy-induced PN at baseline was not an exclusion criteria, as well as progression of myeloma during lenalidomide treatment. *Results.* Of the 34 patient in lenalidomide therapy, 19 (mean age 66 yrs + 8) were available for evaluation with at least six months follow up, 12 of whom at one year. Fifteen patients presented chemotherapy-induced PN at baseline, confirmed also by neurophysiological studies. Four patients had no CIPN at baseline (TNSc: 0) despite previous exposure to bortezomib in three cases, and bortezomib and thalidomide in one case. The majority of evaluable patients (75%) had a TNSc >2 at baseline (median = 6.5, SD ±3.4, range 3-15). At six months, haematological response was documented in 17/19 patients, whereas two patients were in disease progression; at one year all 12 patients showed haematological response. Neurological evaluation revealed improvement (7 patients) or stability (11 patients) of symptoms and TNSc after six months, that persisted unchanged in 11/12 patients at one year. The only patient whose TNSc had worsened at six months, improved to baseline value after 12 months. The improvement of symptoms was more evident in patients with the highest baseline TNSc. All patients with TNSc score 0 at baseline, remained unchanged after six months (3 cases) and 1 year (one case) of lenalidomide therapy. Electrophysiological findings not always paralleled clinical improvement. NRS and ECOG performance status did not change during lenalidomide therapy. *Conclusions.* Using validated clinical tools, the preliminary results of our prospective study suggest that lenalidomide therapy does not worsen and in selected cases may improve CIPN, regardless of MM response.

1473

ACTIVATED PROTEIN C RESISTANCE (APC-R) ANALYZED AS A CONTINUOUS VARIABLE IS DECREASE IN PATIENTS WITH MULTIPLE MYELOMA (MM) AND BENIGN MONOCLONAL GAMMOPATHIES (MGUS) COMPARED TO NORMAL INDIVIDUALS

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Background. Multiple myeloma (MM) patients have an increased risk

of venous thromboembolism (VTE), particularly when exposed to immunomodulatory drugs. Large epidemiological studies have recently showed that patients with MGUS also have an increased risk of VTE compared with normal subjects. Acquired activated protein C resistance (APC-R) is a significant independent risk factor for VTE in hematologic malignancies. *Methods.* We reviewed the records of patients with MM and MGUS for APC resistance by prefakit APC-R test. Patients with documented Factor V Leiden mutation were not included in this analysis. The Prefakit APC-R is a plasma-based functional clotting assay reported as a ratio of patient clotting time with and without APC which is standardized and reported as normalized ratio (normalized to results obtained on pooled normal plasma which is performed on each run). *Results.* APC-R results from 23 MGUS and 73 MM patients were compared with 39 normal subjects. The median APC-R for MM, MGUS and normal subjects were 0.99, 0.90 and 1.1 respectively. MM patients compared to normal subjects, had significantly lower APC-R (P=0.0007). MGUS also showed lower APCR when compared to normal subjects (0.0009). No significant difference was observed between and MM and MGUS (P=0.47) (Figure 1). Baseline characteristics from the three groups were similar in terms of age, sex, and performance status. *Conclusion.* APC-R measured as continuous variable shows a statistically significant decrement in patients with paraproteinemias compared to normal subject and correlates to the underline hypercoagulability observed in patients with MGUS and MM.

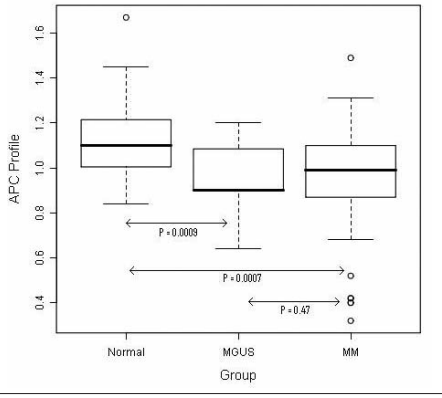

Figure.

1474

EFFICACY AND SAFETY OF LENALIDOMIDE IN PATIENTS WITH MULTIPLE MYELOMA COMPLICATED BY EXTRAMEDULLARY PLASMACYTOMAS

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Background. The role of lenalidomide for the treatment of multiple myeloma (MM) with extramedullary myeloma (EM) is still under investigation. *Aims.* This multicenter retrospective study evaluated the response rate and toxicity profile of lenalidomide-based regimens in myeloma patients with extramedullary involvement at relapse or progression. *Methods.* We reviewed the records of patients treated with lenalidomide-based regimens from October 2007 to February 2010 (n=17, median age 67 years; range 54-87; 8 women). The response of MM was evaluated according to international criteria and the response of EM was evaluated as size changes by physical examination, CT scan or MR imaging. Adverse events were graded with the WHO toxicity scale. The M-protein type was IgG in 9 cases, IgA in 6 and light chain in 2. The type of light chain was lambda in 10 patients and kappa in 7. In 9 patients soft-tissue plasmacytomas developed from underlying bone lesions in the skull (n=3), rib cage (n=4) and paravertebral sites (n=2). Two patients had subcutaneous nodules and 6 had visceral involvement in the liver (n=2), lung and kidney (n=1), periorbital tissue (n=1), cavum (n=1) and pleura (n=1). Multiple localizations were present in 6 patients (35.3%). The median number of prior chemotherapy regimens was 3 (range 1-8), including autologous stem cell transplantation in 3 patients, bortezomib-containing regimens in 16 (94.4%) and exposure to thalidomide in 1. Twelve patients received a standard lenalidomide dose (25 mg/day every 4 weeks) plus dexamethasone (40 mg/d p.o., 1 to 12 doses/cycle) every 3 weeks, and 5 received different schedules. Involved-field radiotherapy was given in 4 cases. 29.4% of patients required lenalidomide dose reduction because of toxicity or intolerance. *Results.* Median duration of lenalidomide treatment was 3.2 months (range 1-15). One case was not evaluable for response because of early death. MM responded to lenalidomide in 11 out of 16 evaluable patients (68.7%). Complete response occurred in 4 (25%) and partial response in 7 (43.7%). Median time to response was 46 days (range 19-363). Regarding EM, tumor size decreased in 11 patients and complete disappearance occurred in 8 (47%) of these. Response of EM was also achieved in 73.3% of patients previously exposed to bortezomib. Median follow-up was 121 days (range 30-474). Median overall survival from the start of lenalidomide therapy has not yet reached; at the time of writing 13 patients (76.4%) are alive and 10 are still on therapy. Only 1 of the 11 responding patients has relapsed. Toxicity profile (grade 3/4) was: thrombocytopenia, 5 (29.4%); anemia, 5 (29.4%); neutropenia, 5 (29.4%); neutropenic fever, 1 (5.8%); others, 3 (11.8%). No deep venous thrombosis was reported but thrombosis prophylaxis was used in most cases (94.1%). *Conclusions.* Results from one of the first studies to specifically evaluate the activity of lenalidomide in EM suggest that lenalidomide-based regimens may be a promising alternative in heavily treated MM patients with extramedullary disease. Further analyses and longer follow-up times are needed to determine the duration of response and the best regimen or combination.

1475

A RANDOMISED CONTROLLED TRIAL OF AN EDUCATIONAL BOOKLET FOR MULTIPLE MYELOMA PATIENTS WITH PERIPHERAL NEUROPATHY

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Background. As overall survival improves in multiple myeloma (MM) patients, quality of life and symptom management are becoming paramount. Peripheral neuropathy (PN) is a debilitating side effect of MM and its treatment but can be managed by monitoring symptoms and changing medication. *Aims.* Improve patient understanding and management of PN in MM patients using an educational booklet. *Methods.* MM patients diagnosed for at least 1-year were randomised (1:1:1) to three treatment groups; Group 1 (control) received no information on PN. Group 2 and 3 received an educational booklet designed specifically for this research project providing in-depth information on PN. Questionnaires were completed at 0, 4, 8 and 12-months to assess impact of educational booklet. Groups 1 and 2 completed the questionnaires themselves and group 3 had the questionnaire completed as part of a telephone interview. Due to a high drop rate, groups 2 and 3 were combined for the analysis. *Results.* 58 patients were enrolled (19 group 1, 39 group 2 and 3). 63% of patients were male, 67% aged >60years and 51% received ≥3 prior lines of therapy with 38-43% of patients on active treatment at any time point. Only 11% had a resolution of their PN over the course of the study but the percentage of patients discussing PN declined by 35%. At baseline, 35% of patients had nothing done to their MM treatment to manage their PN, this increased to 75% at 12-months. In addition, 46% were still not receiving any treatment recommendations to manage their PN symptoms. 46% of patients found it a lot more or somewhat more difficult to be optimistic or hopeful about their progress and survival when they have PN. The educational booklet was reported as good/very good by virtually all the patients, with at least 50% referring to it between 1-3 times at each time point. All patients reported the booklet as easy to understand and the most useful section was on the treatment of PN. 60% of patients think a booklet on PN should be available at diagnosis or at the beginning of their MM treatment with the majority (93%) of patients stating that it should be provided by their Dr or nurse. Over the course of the study, level of knowledge of PN as measured by the correct identification of symptoms associated with PN did not significantly increase. At 12-months, 7% believe that PN is reversible and a further 46% reported that the reversibility of PN depends on the MM treatment that caused the PN. Consequently, 46% of patients stated that PN is not reversible and once you have the symptoms that they will not disappear. *Summary/Conclusions.* Results show that MM patients have a relatively high level of knowledge of the symptoms but not the management of PN,

which was not impacted by the educational booklet. This study shows that PN is impacting on MM patients lives and it is not being managed effectively either through modification of the patients MM therapy or additional treatment recommendations.

1476

SECOND AUTOLOGOUS STEM CELL TRANSPLANTATION AS SALVAGE THERAPY IN RELAPSED MULTIPLE MYELOMA: EXPERIENCE FROM A SINGLE INSTITUTION COHORT OF 28 PATIENTS

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Background. Double autologous stem cell transplantation (ASCT) is widely used as part of the multi-step first-line treatment in multiple myeloma (MM). On the contrary, few data are available as long as the potential utility of second ASCT as salvage for relapsed MM is concerned. *Aims.* To retrospectively investigate therapeutic results from a series of 28 patients in whom second ASCT was used as treatment for relapsed MM. *Methods.* Twenty-eight MM relapsed patients (M/F= 18/10), median age 55 years (range 37-70), received a second ASCT. All but 2 patients of this series were in first relapse. In 23 cases ASCT was performed after a previous salvage regimen (which depended on first line therapy and on the period of relapse, and included chemotherapy, radiotherapy, thalidomide or bortezomib based combinations in 10, 2, 6 and 5 cases, respectively), while 5 patients with a long duration of previous remission were given ASCT as upfront salvage therapy. Between the 23 patients salvaged before second ASCT, status at transplantation was complete remission (CR) in 10/23 cases, partial remission in 12/23, and no response in 1/23. Median duration of previous remission was 24 (3-57), median interval between the two transplants was 25 months (6-65). Median number of $CD34^+$ cells$\times10^6$/kg infused was 5,4 (2,5-23,65) and they had been collected in the mobilization for the first ASCT in 26/28 cases. Conditioning regimen was Melphalan 200, Melphalan 140, and BEAM in 9, 11, and 8 cases, respectively. *Results.* There was no transplant related mortality. All patients were given G-CSF in order to shorten the period of aplasia (one dose of peg-filgrastin in 15 cases, filgrastim for a median of 8 days in 13 cases). Median days to neutrophil $>0,5\times10^9$/L (all patients) and platelet $>20\times10^9$/L (19 patients) were 11 (8-18) and 12 (9-21), respectively. Six patient experienced FUO requiring intravenous antibiotic therapy for a median of 4 days, while there were no case of documented infections. Oral mucositis represented the only relevant extra-hematologic toxicity, requiring total parenteral nutrition in the 5 patients with WHO grade 3 stomatitis. After ASCT, a further improvement of therapeutic results was observed in 13 patients in partial remission (PR); more in detail, 5 patients achieved CR, while 8 ameliorated the degree of PR. In 2 patients a maintenance with thalidomide was started. With a median follow up of 25 months (6-81) after ASCT, 21 patients have progressed, median progression free survival (PFS) 15 months (Figure 1).

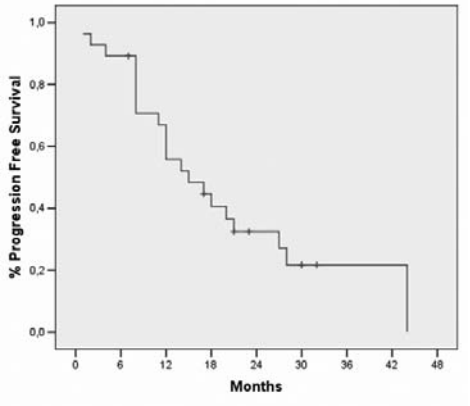

Figure 1.

At the time of writing, 13 patients are alive, while 15 patients died, all of progressive disease; the median overall survival (OS) from second ASCT is 32 months. Duration of previous PFS and interval between the two transplants more or less than 24 months were related to duration of second PFS (P=0.05 and 0.03, respectively). *Conclusions.* Second ASCT is an effective treatment in relapsed MM. Toxicity is similar to that observed when ASCT is executed as first line treatment. Best results are achieved in patients with a duration of previous remission longer than 2 years.

1477

CONVENTIONAL RADIOGRAPHIC SKELETAL SURVEY VS WHOLE BODY MAGNETIC RESONANCE IN MULTIPLE MYELOMA PATIENTS. EXPERIENCE OF A SINGLE INSTITUTION

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Introduction. Conventional radiographic skeletal survey (SS) has been considered the gold standard test for detection of bone lytic lesions in Multiple Myeloma (MM). New imaging techniques as whole body magnetic resonance (WB-MR), have been introduced in the staging of MM because of increased sensivity and specificity of bone lesions. The new updated Durie/Salmon plus system uses MR imaging for a more accurate diagnosis and staging of MM patients. *Aim.* To compare SS and WB-MR images in patients diagnosed of MM in our single institution. *Materials and methods.* A descriptive and retrospective study was performed in 15 patients with MM diagnosed in different Durie/Salmon stages (stage I: 7 patients, stage II: 2 patients, stage III: 5 patients and 1 patient with plasmatic cells leukemia) in our institution from 2001 to 2009. 12/15 patients were chemotherapy-naive. SS and WB-MR were carried out simultaneously for every patient. *Results.* The SS and WB-MR were concomitantly positive for bone lesions in 8/15 patients and were normal in 3/15 patients. In 6 patients with pathological images, WB-MR detected more bone lesions than SS and in 2 patients, WB-MR showed medullary and extramedullary plasmacytomas that SS hadn't detected. In four patients, SS and WB-MR provided discordant results; WB-MR of 2 patients revealed myelomatous lesions in areas where SS was normal leading then to treatment because of stage's change. In the other two patients, WB-MR results indicated a non-myelomatous origin of observed SS lesions. *Conclusion.* Although SS still remains the gold standard imaging technique in the staging of MM patients, WB-MR adds useful new information for staging and treatment decisions. In our experience, 74% of results of both techniques were comparable but WB-MR was superior to SS for detecting more bone lytic and extramedullary lesions.

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BORTEZOMIB, MELPHALAN AND DEXAMETHASONE (VM-DEX) IS A SAFE AND EFFECTIVE THERAPY FOR NEWLY DIAGNOSED ELDERLY PATIENTS WITH MULTIPLE MYELOMA

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Background. The VISTA trial showed great efficacy and superiority of VMP (bortezomib, melphalan and prednisone) *vs.* MP (melphalan, prednisone) in patients ineligible for high dose therapy. Since bortezomib (V) is usually administered with dexamethasone (Dex) in many combination treatments for myeloma, with additive efficacy, the rationale of combining V with Dex and melphalan (M) is immediate and it was tested in elderly MM patients at diagnosis. Although in the past M-Dex was compared with MP resulting in an unacceptable rate of extra-hematological toxicity (mainly pyogenic infections), the rate of complete responses was strikingly superior with M-Dex compared to MP (22% *vs.* 5%). Aim of this study was to test safety of VM-Dex by reducing Dex doses and related toxicity, nonetheless to investigate efficacy of VM-Dex based on this novel combination. *Patients and methods.* In this study 15 patients with a median age of 70 years have been treated with V at usual doses of 1,3mg/m^2 day 1,4,8,11,22,25,29,32 every 42 days - cycle (cycles 1-4) , and day 1,8,22,29 (cycles 5-9). Melphalan was given at 9 mg/m^2 day 1-4 every 42 days- cycle. Dexamethasone was given iv 20 mg on V administration days. *Results.* Thirteen/15 patients completed the treatment. The median number of cycles received was 8. Median follow up is 11 months. VM-Dex therapy resulted in 13/15 objective responses for an overall response rate (ORR) of 86% (CR 7, 47%; VGPR 2, 13,5%; PR 4, 26%). Grade 3-4 hematological toxicities were present in 15% of the patients; more importantly no one experienced bacterial infections/pneumonia. *Conclusions.* VM-Dex combination is a safe and extremely effective combination in elderly untreated MM patients. Larger phase III trials are needed to confirm these data.

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DE NOVO EXTRAMEDULLARY PLASMACYTOMA DURING THE COURSE OF MULTIPLE MYELOMA, A KOREAN SINGLE CENTER EXPERIENCE

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Background. Extramedullary plasmacytoma (EMP), solitary plasmacytoma of bone and multiple myeloma (MM) are identifiable as separate entities by distinct clinical features and natural histories. However, some MM patients who had no plasmacytoma at diagnosis would develop *de novo* EMP during the disease course. Few reports have shown the clinical features of this *de novo* secondary EMP. *Aims.* We aimed to investigate the clinical characteristics and prognosis of the MM patients who developed *de novo* secondary EMP during the clinical course. *Methods.* The records of MM patient registry in Asan Medical Center were reviewed. Between March, 1998 and August, 2008, 31 out of 415 patients (7.5%) with MM who met the criteria of *de novo* secondary EMP were found. None had plasmacytoma at diagnosis. *de novo* secondary EMP was detected by imaging techniques (CT, MRI or PET), and when possible confirmed by histologic study. *Results.* The study cohort of 31 patients showed median age of 51 years (range 33-77 years) at MM diagnosis. Eighteen patients were males. The M-protein isotype were IgG 14, IgA 5, IgD 5, free kappa 4, and free lambda 2. The ISS stage was I in 9 patinets, II in 14 patients, and III in 8. The median number of treatment regimen before EMP was one (range 0-4) and 24 patients received VAD chemotherapy as the first-line chemotherapy. Twenty-two patients (71%) underwent single or tandem autologous stem cell transplantation before EMP development. The site of plasmacytoma included various bones, nasal cavity, obit, stomach, liver, and lung with the most common site of spine. Seven patients (22.6%) had involved multiple sites. The median time from MM diagnosis to EMP development was 31.9 months (range 6.5-75.7 months) At EMP detection, only 6 patients had >25% increase in paraprotein levels. Following *de novo* secondary EMP, 14 patients were treated with local radiotherapy, 9 patients received chemotherapy, and 5 patients received combination of radiotherapy and chemotherapy. Other 3 patients got supportive care. Systemic therapy regimens were VAD in 6 patients, thalidomide in 4, velcade in 2 and others in two. In study cohort, the median overall survival (OS) from MM diagnosis was 39.3 months (95% CI, 28.0-50.5 months) The median OS from secondary EMP was 5.1 months (95% CI, 4.0-6.2 months) and the median progression free survival (PFS) from EMP was 3.5 months (95% CI, 2.8-4.2 months). In the radiotherapy and chemotherapy group, the median OS from EMP was 4.5 and 6.0 months (P=0.535) and median PFS from EMP was 3.0, 3.7 months (P=0.598). Survival was not different among various systemic therapy modalities. *Summary/Conclusions. De novo* secondary EMP would develop in MM patients during their disease course not infrequently (around 8%). The median time to secondary EMP was 32 months following initial diagnosis. The prognosis following *de novo* secondary EMP was very poor regardless of treatment modalities. Novel therapeutic approaches should be explored to improve the outcome of *de novo* secondary EMP.

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DEVELOPMENT OF HIGH-GRADE B-CELL NEOPLASMS AND EXTRAMEDULLARY MYELOMA MANIFESTATIONS FOLLOWING THALIDOMIDE THERAPY

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Thalidomide targets marrow stromal cells, alters IL-6 and TNF-α production and decreases adhesion of malignant plasma cells. Due to modified cell-to-cell contacts and interactions within marrow microenvironment, malignant plasma cells may become resistant to therapy and tend to disseminate and infiltrate other peripheral tissues. Of 93 multiple myeloma patients on thalidomide 7 developed extramedullary symptoms of plasmocyte proliferation: 1st. A 54-year-old man, with IgGκ myeloma from 2004. VAD and EDAP resulted in partial remission. He received thalidomide 100-400 mg/d from April to September 2006, when submandibular lymph node enlargement appeared, composed of blastic $CD38^+$, $CD20^-$, $CD3^-$, $CD79a^-$, $CD56^-$, cyclin $D1^-$ plasmocyte infiltrates with high 90%MIB1. Marrow plasmacytosis was 0.5%, M-protein- 2.63 g/dL. In November 2006 M-protein increased to 4.9 g/dL. He progressed and died in January 2007. 2nd. A 43-year-old man with IgAλ myeloma IIIA. Since December 2008 to September 2009, he received thalidomide 100 mg/d (8 CTD) with partial response. In September 2009 disease progressed and manifested by osteolysis progression, M-protein increase, β2M-6.7mg/L, 98% marrow plasmacytosis (atypical plasmocytes), chest soft tissue tumors composed of large anaplastic cells with acidophil nucleoli and sparse, very large mononuclear or multisegmented-nuclei cells. These *vs.* $CD38c^+$cells were $PAX^{+/-}$, $CD138^-$, $CD56^-$, LCA^-, $CD20^-$, $CD79a^-$, with 10% MIB1. PAD, MP, EDAP were ineffective; patient died in January 2010. 3rd. A 60-year-old IgGκ myeloma patient, from December 2001 treated with VAD, VMCP, VBAP, followed by thalidomide (CTD; May-August 2006), without response. In January 2007 disease progressed, with cutaneous plasmacytomas formation (histopathologically: atypical plasmocytes). 4th. A 62-year-old man with IgGλ myeloma (90% BM plasmacytosis, IgGλ 6.7 g/dL, osteolysis). Thalidomide (4 CTD; December 2008 -April 2009) resulted in partial response. In April 2009, extramedullary disease progression with chest soft tissue tumors was treated with PAD. In June 2009, patient progressed (IgGλ 0.9 g/dL, WBC 55×10^9/L, renal failure) and died. 5th. A 57-year-old IgAλ myeloma woman, on thalidomide (6 CTD; January-August 2009) with partial response, in 8th therapy month developed fulminant plasma cell leukemia (PCL) (4.3×10^9/L of $CD38^{++}$, $CD138^+$, $CD56^+$, $CD54^+$, $CD49^+$, $CD29^+$, $CD126^+$, $CD11a^-$, $CD18^-$ plasmocytes in blood; 70% marrow blast cells with identical immunophenotype) with cutaneous plasmacytomas, renal failure and 1.0 g/dL -13 g/24h urine BJλ. 6th. A 71-year-old man with BJλ-myeloma from April 2005. VAD, melphalan, VMBCP resulted only in disease stabilization. On thalidomide 100-300 mg/d (March-August 2006), he developed PCL in 6th therapy month (26% $CD38^{++}$, $CD138^+$, $CD56^-$ plasmocytes in blood) with hypercalcemia, kidney failure and 3.25 g/dL urine BJλ. Mixed aplastic/hypercellular marrow, was heavily (80%) infiltrated by $CD138^+$ plasmocytes. 7th. A 50-year-old man with IgGκ myeloma infiltrating Th3 vertebra, from June 2004. No remission was achieved after laminectomy, spine irradiation, VAD and EDAP. On thalidomide (200-400 mg/d; May-November 2006), after 2 months, marrow plasmacytosis decreased from 93% to 4% and M protein from 4.7 g/dL to 2,0 g/dL. In November 2006 PCL developed (blood: 40% $CD138^+$, $CD56^+$ plasmocytes; marrow: 83% plasmacytosis. Our findings suggest, thalidomide is effective in initial reducing more mature plasmocytes confined to marrow and allows relatively immature myeloma cells to escape marrow microenvironment.

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ANTITUMOR ACTIVITY OF BORTEZOMIB RETREATMENT IN RELAPSED OR REFRACTORY MULTIPLE MYELOMA PATIENTS

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Background. Bortezomib is a proteasome inhibitor with multiple effects on myeloma cells and bone marrow microenvironment. Bortezomib therapy has become an important part of the standard of care for patients with relapsed/refractory multiple myeloma. Additionally clinical evidences suggest that bortezomib retreatment in patients previously treated with the drug may prolong disease control. Objective. The aim of this retrospective study was to describe patterns and response on retreatment with bortezomib-based regimens on patients with multiple myeloma (MM). *Methods.* Data were retrospectively extracted from the medical records of patients treated in Russia Novosibirsk Hematology Center, who were subsequently retreated off protocol with bortezomib-based therapy. Analyzed patients had 60 or more days between treatments and ≥ 4 bortezomib cycles during initial treatment. Response was evaluated as very good partial response (VGPR) in case ≥ 90% M-protein decrease; partial response (PR) in case 50%-89% M-protein decrease; and minimal response (MR) in case M-protein decrease was less than 50%. *Results.* Retreatment response data were available for 26 patients: 3 (12%) had VGPR, 6 (23%) had PR, 8 (31%) had MR, 7 (27%) had diseases progression and 2 (7%) died. Thus, the overall response rate for bortezomib retreatment was 64%. During retreatment, 15 (58%) patients received bortezomib in combination with another antineoplastic agent, 11 (42%) patients received bortezomib as monotherapy. Median time between bortezomib treatments was 7.9 months; 31% of patients received non-bortezomib maintenance therapy between treatments. Toxicity was a reason for retreatment discontinuation in 27% of patients; the majority of cases (15%) were connected with neuropathy. *Conclusion.* Thus, bortezomib retreatment appears to be safe and effective. Favorable observed response rates with bortezomib retreatment suggest that it may be a viable option for relapsed/refractory multiple myeloma treatment, even in patients previously exposed to bortezomib.

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EFFICACY AND SAFETY OF DARBEPOETIN ALPHA IN KOREAN PATIENTS WITH MULTIPLE MYELOMA WHO ARE UNDERGOING CHEMOTHERAPY: A RETROSPECTIVE MULTICENTER STUDY

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Background. Anemia is one of the common symptoms in multiple myeloma patients that determine the quality of life and compliance to chemotherapy. Darbepoetin alpha is a unique erythropoietin with a longer half life and greater biologic activity than other erythropoietin agents. *Aims.* We decided to evaluate the efficacy and toxicity of darbepoetin alpha in patients with multiple myeloma who are undergoing chemotherapy in clinical practice. *Methods.* We reviewed the clinical data of patients with multiple myeloma who received recombinant erythropoietin during his/her chemotherapy. Efficacy of treatment was assessed using the hemoglobin level and the frequency of transfusion. After 4 weeks of administration of darbepoetin alpha, the increment of hemoglobin more than 2 g/dL from the baseline in the absence of RBC transfusion was defined as the complete response and increment of hemoglobin between 1 g/dL and 2 g/dL or no more transfusions were defined as partial response. Adverse events during the treatment were evaluated by descriptive manner. *Results.* 59 patients were enrolled from 9 centers in South Korea. Of these, 23 were males and 36 females, median age was 67 years (range, 34-85). 5 patients had stage IIA, 1 had IIB, 44 had stage IIIA, and 9 had stage IIIB disease by Durie-Salmon (DS) stage. 44 of 59 patients had heavy chain and others had light chain disease as DS stage system. 36 patients received darbepoetin alpha with their first line of chemotherapy. Responses to the chemotherapy were evaluable in 57 patients. Median dose of darbepoetin alpha was 2.00 ug/kg/week (range 0.88-4.0; 95% confidence interval 1.78-2.01). 36 patients achieved complete response and 9 patients had partial response giving a total response rate of 76.3%. The dose of darbepoetin alpha was not correlated with the degree of hemoglobin increment (P=0.439). Sex, stage at diagnosis, response to concomitant chemotherapy, and previous history of radiation therapy had no effect on the correction of anemia by darbepoetin alpha. Side effects were minimal. Diarrhea and hypertension were reported in 2 patients, respectively, and thrombotic event developed in only 1 patient and resulted in the interruption of administration of darbepoetin alpha. *Conclusions.* Darbepoetin alpha significantly increased hemoglobin and reduced red blood cell transfusions in patients with multiple myeloma receiving chemotherapy with a favorable safety profile. We need to determine the optimal dose of darbepoetin alpha in our patient population by prospective study to avoid unnecessary overdosing.

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THE SIGNIFICANCE OF TWO DIFFERENT FREE LIGHT CHAIN IMMUNOASSAYS FOR MONITORING OF DISEASE ACTIVITY IN MULTIPLE MYELOMA PATIENTS

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Background. Quantification of monoclonal immunoglobulin light chains (FLCs) in the serum is increasingly used in the clinical practice for the diagnosis, prognostic assessment, and also for treatment monitoring of monoclonal gammopathies as an adjunct to standard serum protein electrophoresis and immunofixation. This method is based on the binding of monoclonal antibodies to FLC epitopes that are only exposed if the light chain is not bound within a complete monoclonal immunoglobulin (M-Ig) molecule. It was published that serum FLC assays can be used to follow the disease course in nearly all multiple myeloma patients (Mead *et al.*, 2004). *Aims.* The main purpose of our study was to check the significance of free light chains determination for monitoring of disease activity in multiple myeloma patients. The second aim was comparison of two different FLC immunoassays in this setting. *Methods.* All serum test samples were obtained from our MM patients after receiving their written information consent. Serum M-Ig concentration we measured using electrophoresis gel densitometry or capillary electrophoresis. For determination of free light chains we used immunoturbidimetric measurement by the FreeLite kit and also the new ELISA method performed by kit BioVendor. Manufacturers' instructions were followed without modifications. *Results.* We present several examples of disease monitoring using serum M-Ig and FLCs determination together. In some cases we obtained different information which we had to correlate with other laboratory markers of disease activity and clinical status. A theoretical advantage of serum FLC monitoring is the short serum half-life of FLC compared to intact immunoglobulins. This is the reason for possible use of FLCs determination for detection of early response to therapy which we demonstrate in some our patients. In responding patients FLC levels rapidly fall and stabilise within the normal range. Nevertheless, the reliable measurement of FLC concentration is always hampered by several factors, including crossreactivity of antisera against FLCs, lack of standardisation of FLC quantification and occasional false negativity of FLC measurement despite of positive immunofixation electrophoresis. The results obtained using two different immunoassays for FLCs determination were similar. *Conclusions.* For most patients with a monoclonal immunoglobulin, measuring FLC is unlikely to have additional benefit for monitoring of disease activity with the notable exception of those with nonsecretory/oligosecretory myeloma. FLCs monitoring could identify patients with resistance to current therapy regimens more quickly than M-Ig determination. Conversely the most rapidly responding patients may have a worse outcome. FLCs measurement can help us also in patients with early relapse still on chemotherapy or in case of free light chains escape phenomenon. Because of the kappa/lambda FLC ratio calculation is strongly influenced by measurement errors, therefore we recommend the preferential use of relevant FLC concentration values.

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RELATIONSHIPS OF CLINICAL PARAMETERS AND EFFECTS ON PROGNOSIS OF GENOMIC POLYMORPHISMS IN NOS3, TNF-α AND MDR1 IN PATIENTS WITH MULTIPLE MYELOMA

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In this study, relationships of clinic parameters and effects on prognosis of genomic polymorphisms in NOS3 (+894, VNTR), TNFα (-308, -238 and -857) and MDR1 (C3435T) were assessed in patients with multiple myeloma. Seventy-seven patients with multiple myeloma in the Section of Hematology at the Department of Internal Medicine at Gaziantep University Hospital were included. 77 healthy people without any hematological malignancies were included as control group. We studied these polymorphisms using the PCR and/or PCR-RFLP method. No significant relationship was found between TNFα (-238 and -857) and MDR1 (C3435T) gene polymorphisms and MM. GG genotype in TNFα (-308) gene polymorphism (P=0.012) and TT genotype in NOS3 (+894) gene polymorphism (P=0.004) were significantly increased in patients with MM in comparison with the control group. However, AA genotype in NOS3 (VNTR) gene polymorphism (P=0.007) and GG genotype in NOS3 (+894) gene polymorphism (P=0.004) were significantly decreased in patients with MM in comparison with the control group. In conclusion, TT genotype in NOS (+894) polymorphism and GG genotype in TNFα (-308) polymorphism, which are related to decreased expression, are increased in patients with MM. Thus, this may play a role in the etiopathogenesis of multiple myeloma. Moreover, GG genotype in TNFα (-238) gene is not affected whatever the first-line therapy (OPKHT, bortozomibe, thalidomide) is, but related with early progression not affecting total survival.

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CARDIAC IMPAIRMENT BY AL-AMYLOIDOSIS

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Background. The primary systemic (AL) amyloidosis is a systemic haematological disease belonging to the group of monoclonal gammopathies, characterised by the extracellular deposition of insoluble fibrils which are formed by the fragments or complete molecules of immunoglobulin light chains produced by the clonal plasma cell population, resulting in tissue disorganization and malfunction of the affected organs. Cardiac impairment is usually present by more than a half of the patients and represents the most significant prognostic indicator of this disease. *Aims.* The study aimed at detecting the presence of cardiac impairment by means of laboratory and non-invasive paraclinical methods by patients with AL amyloidosis examined at the time of the diagnosis. *Methods.* The group consisted of 14 patients with the histologically verified AL systemic amyloidosis (12x AL, 2x associated with multiple myeloma), all patients were examined using ECG, echocardiography, levels of NT-proBNP and Troponin T were determined, and some of the patients underwent myocardial magnetic resonance (MRI). *Results.* 10 (70%) out of 14 patients met the criteria of the International Society for Amyloidosis (ISA) for cardiac impairment caused by amyloidosis according to echocardiography; all the patients revealed diastolic impairment and also the electrocardiographic examination showed the characteristic features - namely a decreased voltage in limb leads and QS image in V1-3; an increased level of Troponin T in the serum was recorded in 9 out of those patients. Levels of NT-proBNP were higher by 13 patients, whereas 9 of the patients exceeded 1000ng/l. MRI examination of the heart was positive by 6 out of 8 examined patients who, however, had already shown echocardiographic signs of cardiac impairment. By 2 patients with a negative MRI finding, no ECG or echocardiographic sings of cardiac impairment were detected. *Conclusions.* Preliminary experience confirms the very positive benefit of the implemented examination algorithm of patients with AL amyloidosis, which lies in a combination of imaging methods, heart biomarkers, and together with the ISA criteria, it seems to be the optimum approach in the diagnostics of amyloid cardiomyopathy.

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MULTIPLE MYELOMA IN A 10-YEAR-OLD BOY: A CASE REPORT

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Multiple myeloma (MM), a disease of the elderly, is exceedingly rare below 30 years of age. We present a case of MM in a 10-year-old boy who has been admitted in September 2007 in paediatric unit for a fracture of his left femoral bone during playing rugby. In his history, he's presented a juvenil myelomonocytic leukaemia (JMML) when he was four months old in December 1998. He's treated by aracytine and hydroxyurea during 4 years and he's still in complete response since July 2005. At admission, surprisingly the radiography shows two lytic bone lesions. The RMI finds proximal and distal medullar metaphyso-diaphysairy spreading with broken while previously he had no pain, no symptom. The histology of the two tissue biopsy of the lesion shows some large dystrophic plasma cells, MI 15 positive with no evidence of monoclonal light chains. The bone marrow biopsy shows an interstitial infiltrate of dystrophic plasma cells, with lambda light chain expression. The bone marrow smears finds 5% of dystrophic plasma cells. The monoclonal component IgG Lamda is at 3.56 g/dL, kappa and lambda light chain respectively at 5.65 mg/L and 766 mg/L, the kappa lambda ratio is less than 0.01. There is a proteinuria at 0.64 G per day, haemoglobin at 106 G/L, and Beta2 microglobulin at 2.6mg/L. There is no hypercalcaemia and serum albumin is normal. Plasma Cells Labelling Index is 1,16 % and 6,6 circulating plasma cells/µL in blood. The gene expression profiling can't be made. The PETscanner find multiple uptakes in femoral, vertebral costal and sternal bone. So this boy presents a multiple myeloma with stage 3A Salmon Durie, ISS 1. He undergoes nine cycles of bortezomib (1.3 mg/m^2 D1, D4, D8, D11) and dexamethazone (40 mg/D, D1 to D4) to reach a complete response. An allogenic stem cell transplantation was performed with his sister in the 11th September 2008, with a regimen based cyclophosphamide (60 mg/Kg) and TBI 12Gy, the immunosuppressive regimen is methotrexate (D3, D8, D11) and cyclosporine. He receives a graft containing 3×10^6 CMN/Kg. At Day 120, the chimerism is less than 1% recipient cells, without GVHd, but the monoclonal component reappears. Although he receives only a single cycle of Bortezomib Dexamethazone because of severe neuropathy and gastro-intestinal intolerance, a 2nd response complete has been obtained in June 2009. We report a case of MM during the childhood that is extremely rare. Only very few cases are reported in the literature. In this particular case the boy has been also treated for a JMML that have no relationship with the MM. Infortunaly no cytogenetic or DNA profiling has been made to highlight this feature. At our knowledge it is the first time that such feature is reported. The OS reported by Mayo clinic in a serial of 10 child is 87 months suggesting a better OS that compared in adults.

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BORTEZOMIB PLUS MELPHALAN AND PREDNISONE IN UNTREATED PATIENS WITH MULTIPLE MYELOMA INELIGIBLE FOR STEM CELL TRASPLANTATION

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Background. The latest studies prove that Bortezomib plus Melphalan-Prednisone (VMP) is superior to Melphalan-Prednisone in newly diagnosed patients with multiple myeloma (MM) ineligible for stem cell transplantation, across all efficacy end points, including response rates, time to progression, time to subsequent therapy and overall survival. *Aims.* To evaluate the efficacy and safety of VMP regimen for previously untreated patients with MM who were ineligible for high-dose therapy, in the diary clinical practice. *Patients and methods.* We treated 31 newly diagnosed MM patients from seven eastern andalusian centers with nine 6-week cycles of VMP: bortezomib (1.3mg/m^2 on days 1,4,8,11,22, 25,29 and 32, cycles 1 to 4, and on days 1, 8, 22 and 29,cycles 5 to 9) plus melphalan (9mg/m^2) and prednisone (60mg/m^2) on days 1 to 4, cycles 1 to 9, giving a total of 49 weeks of treatment. Among the study patients, the median age was 71 years (range 59-85) and included 11 male (36%). Disease characteristics at the time of diagnosis were: International Staging System (I 32%, II 45%, III 23%), ECOG performance status <3 (84%), median of plasma cells in bone marrow (20%) and impaired renal function (32%). We assessed the response to treatment using criteria of the European Group for Blood and Marrow Transplantation (EBMT). Adverse events were graded with the use of the National Cancer Institute's Common Terminology Criteria for Adverse Events, version 3.0. *Results.* After a median follow-up of 8 months (1-31) the response rates could be evaluated in 21 patients with a median of cycles administered of 4 (24 weeks). Overall response was achieved by 95% (33% complete response and 62% partial response) and progression 5%.At the data cutoff point, 14 patients (45%) were still receiving the therapy. Toxicity profile was evaluated in all the patients. Adverse events grade 3-4 observed were neutropenia (10%), thrombocytopenia (7%) and gastrointestinal symptoms (13%). Peripheral sensory neuropathy was reported in 51%, including grade 1 neuropathy in 19%, grade 2 in 26% and grade 3 in 7%. 55% of patients required dose reduction of bortezomib, 26% and 11% of melphalan and prednisone, respectively. Ten patients (32%) discontinued treatment because adverse events in 29% and progression in 3%. Time to progression has not reached. The overall survival was 90%. *Conclusion.* VMP is an effective treatment with acceptable toxicity for elderly untreated patients with MM. The response rates and overall survival in our series are similar to data described in previous studies, although a longer follow up is needed in order to confirm these results. The adverse events reported were consistent with established toxicity profiles for both bortezomib and melphalan-prednisone. Therefore, VMP can be considered the standard care in patients who are 65 years of age or older and cannot receive more aggressive treatment.

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AUDIT ON THE USE OF BORTEZOMIB IN CASES OF RELAPSED MYELOMA AT SOUTHAMPTON GENERAL HOSPITAL

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Introduction. Bortezomib is a novel first-in-Class proteasome inhibitor that causes down-regulation of tumour growth and up-regulation of proapoptotic pathways. It also has an anti-angiogenic effect and improves survival in murine models of multiple myeloma. It has been approved for the treatment of relapsed or refractory myeloma by US FDA in May 2003. Since then, it has been recommended as third line therapy in patients with reasonable performance status and organ function and reasonable life expectancy and as an option for the treatment of progressive myeloma in patients who are at first relapse i.e. those who have received one prior therapy and have undergone, or found unsuitable for bone marrow transplantation. *Aims and objectives*. 1) To assess the response of our patients to Bortezomib. 2) To assess the common side effects experienced, in comparison to those reported in the current literature. 3) To assess the survival benefits for patients receiving Bortezomib for relapsed or refractory myeloma. *Methods.* We retrospectively analysed patient notes, e-documents, masterlab- results and pharmacy records looking at patients who received Bortezomib for relapsed or refractory myeloma. *Results.* We identified a total of forty one patients, but three were excluded as they were still receiving ongoing therapy. There was a total of 26 males (68%) and 12 females (32%) with a median age of 62 years (42-62). Their paraprotein profile was IgG in 22 cases, IgA in 6 and light chains only in 8. Most patients (84%) received Bortezomib as a 3rd or 4th line therapy. Eleven patients (29%) required initial dose reduction with a further 10 requiring subsequent dose modifications due to renal impairment, thrombocytopenia and worsening peripheral neuropathy. Nine patients had their treatment interrupted due to herpes zoster in 4, troublesome diarrhoea in 2, a further 2 with pneumonia and one with renal failure. The number of cycles given was more than 4 in 23(60%) patients and less than two in 7(18%). Patients who have shown a good response rate (PP >50% reduction) were 23 (72%) in those who completed at least 2 cycles and 14 (61%) in those who received more than 4 cycles. The overall survival was <6 months in 9 patients (24%), 6 to 12 months in 8 (21%) and 12 to 48 in 2 (5%) *Conclusions.* Limited by its costs, the majority of our patients received Bortezomib as 3rd or 4th line therapy. It was found to be efficacious with reasonable response rates. Its side effects were comparable in our group to those reported but the survival assessment was limited due to the small numbers of patients and short time frame post therapy.

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A STAGED APPROACH WITH VINCRISTINE, ADRIAMYCIN AND DEXAMETHASONE FOLLOWED BY BORTEZOMIB, THALIDOMIDE AND DEXAMETHASONE BEFORE AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION IN THE TREATMENT OF NEWLY DIAGNOSED MULTIPLE MYELOMA

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Bortezomib-based regimens have significant activities in multiple myeloma (MM). In this study, we tested the efficacy of a total therapy with a staged approach, where newly diagnosed MM patients received vincristine/adriamycin/dexamethsone (VAD). VAD-sensitive patients (≥75% paraprotein reduction) received autologous hematopoietic stem cell transplantation (auto-HSCT), whereas less VAD-sensitive patients (<75% paraprotein reduction) received bortezomib/thalidomide/dexamethasone (VTD) for further cytoreduction prior to auto-HSCT. On an intention-to treat analysis, a progressive increase of complete remission (CR) rates was observed, with cumulative CR rates of 48% after HSCT. Seven patients progressed leading to three fatalities, of which two had central nervous system disease. The 3-year overall-survival and event-free-survival (EFS) were 75.1% and 48.3%. Six patients developed oligoclonal reconstitution with new paraproteins. In the absence of anti-coagulant prophylaxis, no patients developed deep vein thrombosis. The staged application of VAD+/-VTD/auto-HSCT resulted in an appreciable response rate and promising survivals. Our approach reduced the use of bortezomib without compromising the ultimate CR rate, and is of financial significance for less affluent communities.

1490

LENALIDOMIDE AS CONSOLIDATION/MAINTENANCE THERAPY IN MULTIPLE MYELOMA PATIENTS

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Background. Lenalidomide is a new immunomodulatory drug with a potent biologic activity. It represents an important treatment option for multiple myeloma patient either as first line therapy, either in resistant/refractory disease or consolidation/maintenance therapy. In this way Lenalidomide increases the available treatment options. *Aims.* According to several studies which evaluated lenalidomide in the treatment of multiple myeloma, in our department, lenalidomide was administered in resistant/relapsing myeloma patients and as consolidation/maintenance therapy in elderly myeloma patients with stable disease (partial remission) after induction therapy or more lines of chemotherapy. *Methods.* We treated 20 patients (11M and 9F) with median age of 70 years (range 66-80). Patients completed almost 12 months of therapy with variable doses of Lenalidomide (5-25 mg/die p.o., according to tolerability of each patient, for 21 days every 28 days), in association of very low doses of dexametasone (10 mg/die p.o. days 1,2,3,4) or alone. We used Enoxaparin for prophylaxis of venous thromboembolisms. Clinical restaging was performed after three, six and twelve months, in course of therapy. *Results.* At the present we didn't observe any progression of disease and in 15 cases we observed a good impact on monoclonal component. In all patients the therapy was well tolerated and were not found significant adverse events. *Conclusions.* Exists a role for lenalidomide with dexametasone or alone for consolidation/maintenance therapy in previously treated elderly myeloma patients. This therapy seems to lead an improvement in prognosis of these patients, without causing severe complications.

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REVERSIBILITY OF RENAL FAILURE IN MULTIPLE MYELOMA PATIENTS TREATED WITH BORTEZOMIB-BASED REGIMENS: A SINGLE CENTRE EXPERIENCE

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Background. Renal failure is a common complication in patients with multiple myeloma (MM). The two major causes of renal insufficiency in MM are myeloma kidney and hypercalcemia. Bortezomib is the first in a new class of pharmacologic agents that inhibit the proteasome. *Aims.* We designed this retrospective analysis to show reversal of renal impairment by bortezomib-based therapy. *Methods.* In this study, eight previously treated patients with acute myeloma induced kidney failure have been evaluated. The median age was 62 years. All patients received bortezomib at a dose l,3mg/m^2 with dexamethasone or in combination with other agents (doxorubicin or melphalan). Two patients required dialysis support at the time of bortezomib administration. *Results.* Three out of the 8 patients (37%) with acute myeloma induced kidney failure experienced reversal of renal failure (complete renal response) and the median time to reversal was 31 days. A partial renal response was documented in two (25%) of patients with the median time to rensponse 48 days. The objective tumor response rate was 75%. All patients who required dialysis became dialysis independent. Toxicities were similar to those seen in myeloma patients without renal failure. *Conclusions.* Bortezomib-based regimens has been shown to be highly effective and to rapidly induce tumor response in patients with multiple myeloma who have renal failure, including those requiring dialysis.

1492

SEQUENTIAL THERAPY WITH VAPLD AND BORTEZOMIB AS INDUCTION THERAPY FOR MULTIPLE MYELOMA: A PHARMACOLOGIC COMBINATION TO ACHIEVE COMPLETE REMISSION BEFORE STEM CELL MOBILIZATION

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Background. Multiple Myeloma remains an incurable disease despite intensive therapy such as high-dose Melphalan and autologous stem cell

transplantation (ASCT), that remains the gold standard therapy. In multivariate analysis achieving complete remission (CR) before ASCT is a significant, independent variable for Overall Survival and Time to Progression. *Aims.* Following this assertion, in a preliminary and feasibility study, we tried to obtain the best response as soon as possible with a combinated and sequential therapy with VAPLD and Bortezomib. In fact using VAPLD or Bortezomib based regimen alone we obtained an Overall Response Rate (CR/VGPR) <50% ; instead in our opinion their sequential use could permit a better tumor mass reduction and the exploitation of synergic role of Bortezomib in front of chemotherapy. *Methods.* From June 06 to September 09 we enrolled 13 patients with untreated MM and 1 pt with relapsed plasmocytoma (previously treated); median age was 53 years (34 - 65), M/F was 3/11; the M-protein type was IgG in 6 pts, IgA in 3 patients, while 4 pts presented a Micromolecular Myeloma (pt with plasmocytoma did not present M-protein at the relapse time); ISS stage was I in 9, II in 3 and III in 2 pts respectively. Patients underwent to VAPLD regimen (Vincristine 1,4 mg/m^2, Pegylated Liposomal Adryamicin (APL) 35 mg/m^2 on day 1 and Dexametasone 40 mg day 1-4) for 3 cycles (day 1-21); 15 days after last therapy, pts started Bortezomib regimen (1,3 mg/m^2 on day 1,4,8,11) for 4 cycles and 30 days after last dose pts received Cyclophosphamide 4 g/m2 and G-CSF for stem cell harvest. *Results.* Response has been evaluated according IMWG uniform response criteria. All patients achieved the partial remission after VAPLD scheme, while OR (CR+VGPR) was achieved in 11/14 (78.5%) patients post Bortezomib. All pts mobilized stem cells achieving target of CD3 4+ (10×10^6/kg) . Toxicity was low and mainly consisted of neutropenia (WHO grade I-II in 15 % of pts) and mild peripheral neuropathy (WHO ≥ grade II in 33% of pts). *Conclusion.* Since our experience was a preliminary and feasibility study, extended up-front therapy with sequential combination of VAPLD, Bortezomib, Cyclophosphamide is more effective in term of remission rate than shorter therapy schedules; moreover it is associated with low toxicity and results in an excellent stem cell harvest. 9 pts underwent to transplant; after ASCT 8/9 patients maintain CR/VGPR, while 1 patient is in RD (median follow-up 16 months, range 2-24 months).

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ARSENIC TRIOXIDE IN PATIENTS WITH REFRACTORY MULTIPLE MYELOMA

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Background. Multiple myeloma is a plasma cell dyscrasia characterized by proliferation of plasma cells in bone marrow associated with the production of monoclonal immunoglobulins. In recent years, the use of, arsenic trioxide, formerly approved for treatment of acute promyelocytic leukemia has been considered for refractory myeloma treatment. *Aims.* This study was designed and carried out to evaluate the efficacy and possible side effects of ATO on patients with refractory multiple myeloma. *Methods.* This study carried out on myeloma patients whose diseases were at least refractory to two standard treatment regimens. Arsenic trioxide was administered as an intravenous infusion at a dose of 0.25 mg/kg/d for 5 d/week during the first 2 consecutive weeks of each 4-week cycle with 2 week rest. Patients who completed one 4-weak cycle were evaluated for response to treatment. *Results.* 12 patients with refractory disease to conventional treatment regimens, received arsenic trioxide. disease assessment was based the amount of serum proteins electrophoresis . of the10 patients; stable disease was observed in four patients (33%), progression disease in five patients (41.6%), complete response in one patient (3.8%) and the reemaning two patients could not be assessed for a response (because of increased liver enzymes after the first week). Some adverse events are: increase liver enzymes and serum creatinine , neutropenia, pruritus, nausea, vomiting, lower extremities edema, noninfectious diarrhea were observed. *Conclusion.* The use of arsenic trioxide is promising in treatment of refractory multiple myeloma.

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OSTEONECROSIS OF THE JAW IN PATIENTS WITH MULTIPLE MYELOMA TREATED WITH BISPHOSPHANATES

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Background. Bisphosphonates are pyrophosphate analogues which inhibit osteoclastic activity. Long term use of bisphosphonates has been recently associated with osteonecrosis of the jaw (ONJ). ONJ is defined as three months non healing defect in a jaw, usually in mandibule. ONJ is commonly precipitated by a tooth extraction or other stomatological procedure in patients treated with long term, potent, high dose intravenous bisphosphonates for the management of multiple myeloma (MM), breast or prostate cancer. Current evidence shows that the risk of ONJ in non-cancerous patients, such as those with osteoporosis, is very low. *Aims.* The aim of this study was to evaluate the incidence of ONJ in patients with MM treated with bisphosphonates in our institution and to report the first two cases. *Methods.* We have analyzed 247 patients with MM diagnosed and treated in our institution in the period of 2002-2009. Annual incidence of myeloma in Republic of Macedonia in that period was 30±10.9 new cases per year. Median duration of bisphosphonate therapy was 24.7 months. We have been used 90 mg of pamidronate or 6 mg of ibandronate i.v. per month or standard dose of clodronate or ibondronate orally. Zolendronate is not available in our institution and we have only two patients treated with zolendronic acid in foreign hospitals. *Results.* We have diagnosed two patients with ONJ from 247 evaluated (0.8%). First case is a female patient 58 years old, diagnosed with IgG myeloma 10 years ago, treated with multiple chemotherapy regiments. First, she received 17 cycles of COMP (cyclophosphamide/vincristine/melphalan/prednisone) and than interferon with lamivudine and prednisone due to aggressive hepatitis B infection. We started with Thalidomide/Prednisone and bisphosphonates therapy in 2004. From August 2004 until October 2009 she received 18 doses of ibandronate i.v. 6mg/per dose and 24 doses of pamidronate i.v 90 mg/per dose. Due to X-Ray signs of ONJ bisphosphonates therapy was stopped in October 2009. She was treated surgically in January 2010 with pathological proof of ONJ. Now she receives only chemotherapy with Melphalan/Prednisone/Thalidomide (MPT). Second case is a male patient 55 years old, diagnosed with IgA myeloma in February 2008. Initially he was treated with four cycles of VAD (Vincristine/Doxorubicine/Dexamethasone), hematological remission was not achieved, so he continued with 4 cycles of COMP. From October 2008 he was treated with MPT. Therapy with oral ibandronate was started in February 2008 and it was stopped due to ONJ in August 2009. The diagnosis was pathologically confirmed. Now he is treated only with MPT chemotherapy. Both patients have regular check-ups for ONJ and condition is slowly improving. *Conclusion.* ONJ is an uncommon but long-lasting complication of long-term treatment with bisphosphonates. Fortunately this complication was rare in our hospital and we have diagnosed only two patients, one treated with oral ibandronate and one treated with i.v. pamidronate and ibandronate. Both patients didn't have stomatological procedure before occurrence of ONJ. Low incidence of ONJ in our institution could be explained with the rare use of zolendronate, which is the most commonly referred bisphosphonate causing ONJ and with relatively shorter duration of bisphosphonate treatment comparing with other studies. Despite the fact that ONJ is a rare complication in our department preventive measures like stomatological examination before bisphosphonates treatment and regular stomatological check-ups during bisphosphonates treatment must be considered.

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MULTIPLE MYELOMA: 68 CASES, PORTUGUESE ONCOLOGICAL CENTER'S EXPERIENCE

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Background. Multiple myeloma (MM) is a disease caused by plasma cell clonal proliferation, and it represents 10% of all hematological malignances. *Aims.* Characterization, determination of the prognostic factors, treatment response and overall survival of the patients diagnosed with MM at Instituto Português de Oncologia do Porto, Portugal. *Methods.* We gathered a series of cases, based on retrospective revision of

clinical data of patients diagnosed with multiple myeloma, at our Institution, between January 2007 and January 2009. Statistical analysis was made on xlstat2009. *Results.* 68 patients were identified (53% male, median 65 years old). Median follow up was 16,5 months. Twenty patients (29%) had international staging system (ISS) 1, 27 (40%) had ISS2 and 21 (31%) had ISS3. At diagnosis, 16 patients (23,5%) had and increased creatinine and 20 patients (29%) had lactic desidrogenase above normal. Median hematological values were: 10,9g/L hemoglobin, $5{,}76\times10^9$ WBC/L, $1{,}82\times10^9$ Lymphocyte/L, 195000 Platelets/L. Median bone marrow plasma cell count was 30%. The most prevalent immunoglobulin was IgG (37patients, 54%), followed by IgA in 18 patients (26%) and light chain in 7 patients (10%). Thirty patients (44%) were submitted to chemotherapy with ID (idarrubicin+dexamethasone), 15 (22%) with MP (melphalan+prednisone), 7 (10%) with MPT (melphalan+prednisolone+thalidomide), 4 (6%) with TD (thalidomide+dexamethasone) and 4 (6%) were enrolled on international investigational protocols. Thirty four patients (50%) had disease progression. Regarding 2nd line treatment, 21 (62%) of them used tacydex (thalidomide+cyclophosphamide+dexamethasone), 3 patients (9%) used bortezomib+dexamethasone and 3 (9%) of them were enrolled in international investigational protocols. Twenty five patients (37%) underwent autologous bone marrow transplantation. Median overall survival of these patients is statistical superior when compared with those who were not submitted to transplantation (wilcoxon P=0,006). ISS shows 3 statistical different overall survival curves. Global overall survival hasn't reached yet the median survival. Creatinine value at diagnosis shows a statistical difference in global survival (wilcoxon P=0,048). Lactic desidrogenase appears to separate two statistical different overall survival groups (wilcoxon P=0,056). Total lymphocyte count doesn't show statistical difference on overall survival (wilcoxon P=0,789). *Conclusion.* The presented case series puts in evidence the changes in adopted treatments, considering recent advances in this field. Overall survival curves are similar to those published in literature, enhancing the importance of autologous transplantation. The ISS, creatinine value at diagnosis and lactic desidrogenase are prognosis factors, in this series.

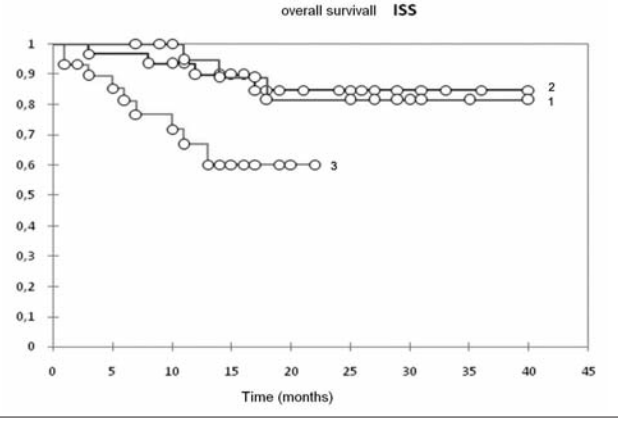

Figure.

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COMBINATION OF BORTEZOMID, CYCLOPHOSPHAMIDE AND DEXAMETHASONE: TREATMENT OF MULTIPLE MYELOMA AND PLASMATIC CELL LEUKEMIA PATIENTS. EXPERIENCE OF ONLY ONE INSTITUTION

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Background. Novel sequential combination therapy for induction may improve the quality of response and therefore prolong survival in newly diagnosed multiple myeloma (MM) patients. This concept should be allocated in refractory / relapse multiple myeloma patients. *Material and methods.* We present our experience with the combination of Bortezomid (1.3 mg/m^2 D1, D4, D8, D11), Cyclophosphamide (600 mg/m^2 D1, D8) and Dexamethasone (40 mg D1-2, D4-D5, D8 - D9, D11-D12) for 21-days cycles in newly and relapsed multiple myeloma and plasmatic cell leukemia patients. From February 2009 until February 2010, we treated 12 patients (7 in first line). On the front line in less than 65 years old as induction therapy prior to stem-cell transplantation, have treated 5 patients (1 plasma cell leukemia included). In first line, over 70 years, two patients with plasma cell leukemia. In the 5 patients in relapse had received at least two lines of prior chemotherapy (2-5), all had received prior Bortezomib, and 1 patient had been transplanted previously. The number of cycles (BCD) administered ranged between 1-6 (mean 2.4). 4 patients received only one cycle, 3 died of disease progression and in newly diagnosed patients stopped treatment for pneumonia (Candida plus H1N1 virus) and he admitted in intensive care unit because he did need mechanic ventilation support. *Results.* 8 patients are evaluable for response; Overall response rate was 100%, including 12.5% CR, and 87.5% partial response. 4 patients treated with RP, they patients have undergone stem-cell transplantation. Full results will be presented the congress. At the moment, 5 patients completed all six cycles. Toxicities were predictable and manageable; the most-commonly reported grade 3/4 toxicity was neuropathy (37.5%).The limiting toxicity was peripheral neuropathy, present in 3 patients, necessitating dose reduction of Bortezomid a step in 2 patients, and manage it in one week. 4 patients (50%) developed neutropenia grade 3-4, and required to use G-CSF. Infectious complications that required hospital admission in 3 patients (1 ZHV spread, bacteremia Staf., 2 pneumonias). *Conclusions.* 1) The BCD combination gets quality and fast respond. 2) hematological toxicity, is easily manageable, and the incidence of infection complications is higher in refractory / relapsed patients and with associated comorbidity. 3) In refractory / relapse patients, respond rate is obtained as first-line patients. Patients retreated with bortezomid had good results, with an increased of neuropathy.

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NEGATIVE ASPIRATION OF SUBCUTANEOUS FATTY TISSUE WITH NEGATIVE ORGAN INVOLVED BIOPSY DO NOT EXCLUDE A DIAGNOSIS OF PRIMARY SISTEMIC AMYLOIDOSIS

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Background. Primary Sistemic Amyloidosis (AL) is the most frequent form of systemic amyloidosis and its morbilility is associated with Ig light chains deposition in vital organs. Early diagnosis and treatment is crucial. We report two consecutive patients with a final diagnosis of AL who had negative aspiration of subcutaneous fatty tissue (ASFT) and initial negative organ-involved biopsy. *Cases reports.* Patient 1: A 56-year-old male consulted our department with nephrotic syndrome and monoclonal G- protein in the serum with a peak of 0.5 g/dL. The complementary exams are showed in Table 1. With a high suspicion of AL, an ASFT with congo red staining was performed being negative for amyloid. Subsequently, a kidney biopsy was carried out but no amyloid or Ig deposition was proved so patient was supposed to have a MGUS. Nephrotic syndrome was treated unsuccessfully with steroids and 10 months later a new kidney biopsy was repeated, showing amyloid deposition. The revaluation showed biological deterioration (Table 1). Orthostatic hypotension appeared and increased wall thickness was evidenced in the echocardiographic examination. These new findings led to a diagnosis of AL. As he had three organs involved, the transplantation was not consider, and he has been treated with Melphalan and Dexamethasone. To date, after four cycles, he has reached partial response. Patient 2: A 76-year-old male suffered from nephrotic syndrome with an Ig G paraproteinemia (0.58g/dL) and 8-months history of anasarca, the "racoon sign"in physical examination and complementary exams showed in Table 1. An ASFT and a kidney biopsy failed to demonstrate amyloid or Ig deposition. Despite these findings, a rectal biopsy was performed, showing amyloid. An echocardiographic examination showed a restrictive cardiomyopathy with ventricular septum hypertrophic. He has been treated with Melphalan and Dexamethasone without response after two cycles. *Discussion.* In both patients, despite the high clinical suspicion of AL, both ASFT and biopsy of the organ involved failed to confirm the diagnosis. First patient suffered from a wrong diagnosis and the second one could only be correctly diagnosed after a rectal biopsy. Amyloidosis diagnosis is determined through histological material from biopsy of different parenchymal organs, which have high diagnostic value, but hide risk of bleeding. ASFT is a safe, simple and fast method for detecting amyloid and its sensitivity is about 60-90%, so this technique can be used as a screening test. However, sometimes ASFT fails so biopsy from a symptom-giving organ is mandatory in cases with high clinical suspicion with negative ASFT. In these cases, although the combination of ASFT and involved organ biopsy has a very high sensitivity, if both techniques are negative, we must perform a rectal or gingival mucosa biopsy, or repeat

the organ biopsy, but never give up, because clinical suspicion, very frequently leads to the diagnosis of AL, and late diagnosis or misdiagnosis could have fatal consequences.

Table. Principal biological findings of the two cases.

BIOLOGICAL FINDINGS:	Patient 1: Diagnosis	Patient 1: evolution	Patient 2
•Creatinine (mg/dl)	0,6	0,8	1,8
•Calcium (mg/dl)	7,6	8,6	8,8
•Albumin (g(dl)	1,5	1,1	2,5
•MC (g/dl)	0,51	0,58	0,9
•Isotipe of MC	Ig G λ	Ig G λ	Ig G λ
•ProBNP (pg/ml)	362,5	4891	6807
•serum Free light chains (ratio)	0,34	0,06	0,14
•Prot urine (g/24h)	23,46	45	9,1
•BJ (g/24h)	0,46 λ	0,26 λ	0,36 λ
•BMPC (%)	3	-	13
•CPa/CP flow cytometry (%)	97	-	88
•Bone radiographies	normal	normal	Skull lesions
•Aspiration of subcutaneous fatty tissue (congo red)	negative	negative	negative
•Renal Biopsie (congo red)	negative	positive	negative
•Rectal Biopsie (congo red)	-	-	positive

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HYPOMETHYLATING THERAPY INDUCES CLINICAL RESPONSE IN MULTIPLE MYELOMA

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Background. Azacitidine, a hypomethylating agent approved in Europe (December 2008) for the treatment of myelodysplastic syndromes (MDS), has demonstrated *in vitro* efficacy in the treatment of multiple myeloma (MM) cells. *Aims.* We present the clinical data of a patient treated with azacitidine for MDS who also showed a response for MM. *Methods.* A 56-year-old man diagnosed with stage-IIIB MM in December 2003 (baseline status at diagnosis: IgA lambda in urine 4380 mg/24 h, severe bone disease and vertebral mass with plasmacytomas, 40% bone marrow infiltration, Bence-Jones protein 500 mg/dL, Hb 9.3 g/dL, platelets 19 500/μL, WBC 9000 cel/mL, calcium 8.9 mg/100 mL, β2M 5625 U; creatinine 2.8 g) was initially treated with VAD (4 cycles), followed by hyper-CVAD (3 cycles) plus radiation therapy, melphalan-prednisone (5 cycles), dexamethasone plus melphalan (1 cycle) and dexamethasone (5 cycles, 40 mg for 4 days). He also received deferoxamine for 1 year for ferritin levels of 3000 ng/mL (starting in April 2006), and interferon-α (from May 2007 to April 2009) for hepatitis C virus (HCV) infection diagnosed in March 2007. From May to September 2009 12 RBC units were transfused for Hb levels of 7 g/dL. The diagnosis of MDS and refractory anemia with ringed sideroblasts (RARS) was confirmed in June 2009 (baseline status at diagnosis: bone marrow trilineage dysplasia, multiple cytogenetic changes, blasts 1%, plasma cells 3%, ringed sideroblasts 40%), and treatment with azacitidine was initiated at 75 mg/m^2 for 5 days in a 28-day cycle.

Table 1.

		Situación inicial (Agosto 2009): MM y SMD (ARSA)					
		Hg (g/dl)	Plaquetas (u/ul)	Neutrófilos (cel/mm^3)	B2M (unidades)	Leucocitos (cel/ml)	Ferritina (ng/ml)
		10,5	32000	800	3000		
		VIDAZA ®					
	Dosis	Hg (g/dl)	Plaquetas (u/ul)	Neutrófilos (cel/mm^3)	B2M (unidades)	Leucocitos (cel/ml)	Ferritina (ng/ml)
Tras ciclo 1	75mg/m^2/5días	8	19000	280			
Tras ciclo 2	37,5mg/m^2/5días Dosis reducida por toxicidad	11,5	108000	1260			
Tras ciclo 3		14,5	120000			6000	
Tras ciclo 4					2000		
Tras ciclo 6		14,2	82000	1940			700

Results. The patient was refractory to VAD and hyper-CVAD therapy plus radiotherapy. Subsequent treatment with melphalan-prednisone, melphalan-dexamethasone and dexamethasone (due to melphalan toxicity) achieved a good response. In November 2005 IgA lambda levels were 1090 mg/24 h. Ferritin levels decreased from 3000 ng/mL to 400 ng/mL after treatment with deferoxamine. Further to HCV-DNA negativization, treatment with interferon was continued because it proved to be beneficial for the patient's MM: IgA levels had diminished to 300 mg/dL (kappa/lambda ratio 0.19) and Bence-Jones protein decreased to 100 mg/24 h. In April 2009 interferon was discontinued due to decreased platelet counts and Hb levels. Following the diagnosis of MDS-RARS, azacitidine therapy was initiated in August 2009. In September 2009 the monoclonal band (IgA λ) was still detectable despite transfusion of 12 RBC units for low Hb (7 g/dL). The following results were obtained (Table 1). The initial dose was reduced to 50% due toxicity from cycle 2. The increased levels of hemoglobin, neutrophils and platelets indicated response of the MDS-RARS. In December 2009 (cycle 6) no monoclonal band was detected, which indicated response of the MM. *Summary conclusions.* Azacitidine elicited a complete response of MDS (RARS) in several studies, but in our patient it also improves the response of previously treated MM. To our knowledge this is the first clinical evidence of the efficacy of azacitidine in MM.

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NEUTROPHILIA IN ASSOCIATION WITH MULTIPLE MYELOMA: CASE REPORT

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Background. Neutrophilia might be present in certain types of solid tumors and hematological malignancies. Case report: We report the case of a 77 year-old woman who was admitted in our hospital due to a one-month evolution constitutional syndrome. As relevant, CBC showed ,71000/μl leukocytes (left shift, metamyelocytes and myelocytes in the blood smear) and 7.5 g/dl of hemoglobin. Biochemistry pathologic values were: creatinine 4.7 mg/dl, Uric acid 10.7 mg/dL, LDH 455U/L, GGT 264U/L and Vit B12>2000 pg/mL. Chest X-ray showed bilateral pleural effusion. An splenomegaly of 17cm was observed in the abdominal ultrasound and CT. The NAP was 145. With the suspected of chronic myeloproliferative syndrome, a bone marrow (BM) biopsy was performed. BM aspiration was hypercellular with granulocytic hyperplasia and 12% of pathologic plasmatic cells (100% showed an aberrant immunophenotype: CD19$^-$/CD56$^+$/CD117$^+$/CD28$^-$, monoclonal kappa). BM biopsy showed myeloid proliferation, and infiltration by kappa monoclonal plasmatic cells. There was also a moderate reticulin fibrosis. The study for BCR/ABL and JAK2V617F was negative. There were no cytogenetic abnormalities. At the same time, we got the blood and urine proteinogram. It showed a serum biclonal component (IgA-kappa of 5.39 g/L and free kappa light chains of 1.80 g/L) and a monoclonal component, in 24-hour urine, of 2.73 g/L with positive inmunofixation for free kappa light chains. The B2M was 19.5 μg/mL. As all these results directed us to a probably Multiple Myeloma (MM), we asked for a total body X-ray, that was normal. Given the association of a suspected MM with leukocytosis, we reviewed the literature related to this issue. As it seems that this association is related to G-CSF production by plasmatic cells, we quantified the mRNA of this gene in the BM. Plasmatic cells were selected by CD138 positive cell separation. The analysis by real-time PCR demonstrated an intense expression of G-CSF by pathologic plasmatic cells. The final diagnosis was a G-CSF-producing IgA-MM. The patient was treated with Bortezomib and Dexamethasone, showing a lessening in the number of leukocytes as well as in the BM infiltration by plasmatic cells. *Discussion.* G-CSF is produced by monocytes, macrophages, endothelial cells and fibroblasts but not by normal plasmatic cells. It has also been demonstrated the production of G-CSF in some solid tumors and hematological malignancies (Hodgkin's lymphoma and anaplastic large cell lymphoma). In the few case-reports describing the coexistence of Monoclonal Gammapathy (MG) and neutrophilia, it was suggested that plasmatic cells might secrete some kind of interleukin or growth factor that stimulates myeloid proliferation. When serum was analyzed, high levels of G-CSF were detected, which was consistent with a study where molecular RNA overexpression of G-CSF was observed in plasmatic cells. We report another case of RNA overexpression of G-CSF in pathological plasmatic cells, which might explain the high neutrophil and myeloid proliferation associated with a primary diagnosis of MM. As the literature describes an association between chronic neutrophilic leukemia and plasma cell dyscrasia, we considered that it might be the result of a G-CSF-producing MM, instead of a real coexistence of both diseases.

1500

THE EVALUATION OF THE EFFICACY COMBINED TREATMENT WITH REVLIMID AND DEXAMETHASONE IN PREVIOUSLY TREATED SUBJECTS WITH MULTIPLE MYELOMA (MM) - THE OWN STUDY REPORT

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Multiple myeloma (MM) is an incurable disease that is characterized by the accumulation of clonal plasma cells in the bone marrow.The primary approach to the treatment of MM is systemic antineoplastic therapy, which increased the median survival but does not allow to achieve sustained disease stabilization. Revlimid is the analogue of the Thalidomide administered to heavily pretreated patients with relapsed or refractory multiple myeloma. The mechanism of anti -myeloma activities are : modulation of the adhesion of myeloma cells to bone marrow stromal cells, cytokine inhibition, antiangiogenesis, immune modulatory effects- induction of a Th1 T-cell response with secretion of interferon-y and interleukin-2 and direct effects on myeloma cells and / or bone marrow stromal cells. The aim our study was to compare the efficacy of oral Revlimid in combination with oral pulse high-dose Dexamethasone in refractory multiple myeloma patients (1-3 lines of therapy). *Materials and Methods.* We randomized 10 MM II-IIIoA-B Durie Salmon stage patients (4 female, 6 male), aged 33-77 who were considered to have disease progression after at least 2 cycles of anti-myeloma treatment or have relapsed with progressive disease after treatment. Patients received Revlimid at a dose 25mg daily for 21 days every 28 days and oral pulse dexamethasone at a dose of 40mg daily on Days 1-4, 9-12 and 17-20 of each 28-day cycle. All dose modifications were due to Revlimid toxicity (3/4 grade NCI). *Results.* We obtained the Plateau Phase of Hematological Response at 5/10 pts.-

Table 1.

Response RR
Number of pts. 1
Since Cycle Number 12
Median Response Time (mo) 6.5
CR -2pts since 4-8 cycle , median response time- 38.6 months
PR- 2pts since 2-4 cycle ; median response time- 5.6 months
S- 4pts ; median response time -50.4 months

Of 7 pts were discontinued because of progression disease (PD). Two patients died of acute heart failure and 4 died of PD. All patients have different side effects (diarrhea, infections, bone and abdominal pain, fever, disorders of consciousness) and the dose of Revlimid was modied at 1 patient. Median progression free survival time was 20.4 months, time of survival since diagnosis was 67 months, and time of survival since Revlimid therapy 36.9 months. *Conclusions.* - Revlimid is the effective oral medicine which induces a high proportion of complete and partial long-lasting remissions in patients with relapsed or refractory multiple myeloma. - Toxicity at patients was acceptable (grade 1-3)

1501

BORTEZOMIB FOR THE TREATMENT OF MYELOMA IN OUR HEMATOLOGY DEPARTMENT

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Background. Bortezomib is a selective proteosome inhibitor used for the treatment of myeloma. Trials demonstrated useful therapeutic effect; however its use was associated with a significant early discontinuation rate due to side effects. *Aims.* The aim was to determinate how Bortezomib was being used in our department: specifically, to determine the number of treatment courses patients are able to complete; to establish clinical affectiveness in multiple myeloma patients; to determine the overall survival and tolerability. *Methods.* Retrospective analysis was done for 27 multiple myeloma patients. We analysed the number of courses, the response rate, the tolerability and the side effects. 16 patients were male, 11 were female; median age 62 (range 45-80). 69% received Bortezomib in combination with Dexamethasone and 21% received Bortezomib in combination with other chemotherapy. 58% of patients received Bortezomib as third line treatment or greater. *Results.*The median number of treatment cycles completed was 4 (range 1 to 8). The complete response rate was 10% and the partial response rate 51%. 39% of patients discontinued treatment early due to side effects: neuropathy, fatigue and gastrointestinal symptoms were the most commonly cited. The median overall survival derived by Kaplain/Meier, method was 14 months. Peripheral neuropathy was the single most frequent side effect necessitating early discontinuation. The combination of Bortezomib with corticosteroids may be associated with higher rates of autonomic neuropathy than single agent Bortezomib. *Conclusions.* Our experience is that Bortezomib is an effective treatment for patients with multiple myeloma and peripheral neuropathy can determine early discontinuation of therapy.

1502

SPECTRAL KARYOTYPING (SKY) REVEALS A NEW SUBSET OF PATIENTS WITH MYELODISPLASTIC SYNDROME (MDS) AND CHROMOSOMAL ABNORMALITIES NOT SEEN IN G-BANDING ANALYSIS

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Background. In myelodisplastic syndrome (MDS) cytogenetic findings are essential for a correct diagnosis and classification of the disease and constitute one of the most important independent prognostic factor. The cytogenetic profile, however, often cannot be fully resolved by banding because of the presence of marker chromosomes, rings, and unidentified material attached to chromosomes. Spectral karyotyping (SKY) has proven to be a powerful tool because it allows for refinement of the interpretation of complex karyotypes in hematological malignancies. AIMS The objective of this investigation were the comparison of SKY with G-banding analysis in order to identify new chromosomal abnormalities "masked"by the limited resolution of classical cytogenetics. METHODS Bone marrow samples of 24 patients diagnosed with MDS were incubated in RPMI 1640 with 20% fetal calf serum for 72~96h at 37 C. Cells were exposed to Colcemid (0.1 μg/mL) for 20min at 37 C and harvested routinely. Metaphase chromosomes were GTGbanded by a conventional Trypsin-Giemsa technique. Karyotypes were described following the recommendations of the International System for Human Cytogenetic Nomenclature. For SKY analysis, slides were freshly prepared from chromosome suspensions stored in fixative (methanol/acetic acid 3:1) at -20 C. *Results.* In a group of 24 cases studied, the cytogenetic analysis (G-banding) showed chromosomal aberrations in 13 patients (54.2%) and normal karyotype was observed in 11 subjects (45.8%). The abnormalities observed were dup(1)(q21q32), inv(3)(q21q26), t(3;3)(q21;q26), +4, del(5)(q31), -7, del(7)(q22q36), +8, add(17)(p12), +i(17)(q10), del(20)(q11). In the group with normal cytogenetic, SKY analysis revealed "masked"chromosomal abnormalities in 6 patients, being t(7;9)(q36;q34), ins(1;6)(q21;?), t(11;12)(p15;q24.1), ins(3;5)(p21;?), t(8;16)(q23;?) and ins(6;11)(q21;?). Among 13 cases studied with previous chromosomal abnormalities by G-banding analysis, SKY identified additional abnormalities in 8 patients. Some abnormalities found include t(6;9)(q27;q22), t(12;17)(p13;p12) and t(8;11)(p12;q12). Although in the majority of the cases the frequency of the abnormal clones was less than 50%, the abnormalities identified by SKY were classified as clonal. In five cases SKY analysis would have changed patient's cytogenetic risk (WPSS from low to intermediate in 3 cases and intermediate to high risk in 2 cases). Conclusions. SKY analysis has proved to be a promising and reliable method for identification of additional and complex chromosomal abnormalities usually present in a great number of human neoplasias. To better define the prognostic contribution of these new chromosomal abnormalities identified by SKY, the analysis of a larger number of patients are necessary.

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1503

MUTATIONAL ANALYSIS OF TET2 GENE IN BCR-ABL1 NEGATIVE CHRONIC MYELOPROLIFERATIVE NEOPLASMS

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Background. It has been recently reported that acquired loss of heterozygosity at chromosome 4q24 (where *TET2* is located) is associated with myeloproliferative neoplasms (CMPN) and myelodysplastic syndromes. Moreover, recent research has also detected mutations in

TET2 in these syndromes. *Aims.* Our purpose is to analyze all the coding region of *TET2* in 11 cell lines derived from patients with different hematological malignancies (MOLT-4, RAJI, K-562, HL60, KARPAS-299, BK0006, BK0013, M07-e, UKE-1, SET-2, HEL) and in 34 patients with CMPN (10 positive and 24 negative for *JAK2V617F*). We also analyzed 20 samples from healthy Caucasians as controls. *Methods.* To perform the mutational analysis of *TET2* we used denaturing High Performance Liquid Chromatography (dHPLC) and sequenced two samples from each elution profile for every fragment. Sequence traces were analyzed using *Mutation Surveyor* and reviewed manually. Statistical analyses to determine putative associations between allele and genotype frequencies of SNPs detected and disease development were carried out with *SNPStats*, two tailed Fisher's exact test and chi-square 2×3 contingency test with two degrees of freedom with two rows. In order to check the potential effect of intronic changes (both SNPs and mutations) on mRNA splicing, we used the splicing prediction web tool *Human Splicing Finder*. *Results.* We detected eight polymorphisms already described in dbSNP, and four not previously described. All of them were found both in control samples and in patients. Furthermore, we detected seventeen mutations not previously reported in two cell lines and nine NMPC patients. Twenty percent (2 of 10) of *JAK2V617F*-positive patients showed mutations in *TET2* (both cases were PV patients), while mutations were found in 29% (7 of 24) of *JAK2V617F*-negative patients. Four of these 7 patients (57%) had more than one mutation in *TET2*. Five of the 17 mutations detected were located in *TET2* regions highly conserved in other *TET* family proteins and also across species. None of the eight polymorphisms already described in dbSNP showed different distribution between patients and controls or between *JAK2 V617F*-positive and -negative patients. *Conclusions.* As it has been previously described by other authors, our results show that *TET2* is frequently mutated in NMPC patients (26.5%). The mutations detected were heterozygous and 13 were potentially inactivating mutations: five nonsense mutations, four missense mutations and four frameshifts. *TET2* exons 4 and 11 were the most frequently affected. Among the seven *JAK2 V617F*-negative patients with *TET2* mutations, five (72%) had a low survival. Further studies are required to understand the biological consequence of these mutations. To confirm that the four sequence changes, detected in control samples and patients, are polymorphisms, we will include more control samples.

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1504

BCR/ABL EXPRESSION IS NOT A RARE EVENT IN PROGRESSION OF CHRONIC MYELOPROLIFERATIVE DISORDERS

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Background. Understanding of the molecular pathogenesis of chronic myeloproliferative disorders (CMDs) was significantly improved after discovery of Jak2V617F mutation, deletions of exon 12 of Jak2 (Jak2-ex12) and MPLW515L/K. Nevertheless, there are still questions concerning the difference of clinical representation of polycythemia vera (PV), essential thrombocythemia (ET) and idiopathic myelofibrosis (IMF) as well as the molecular mechanisms that underlie progression of this group of diseases. In 2007 Dr I Mirza had described two PV cases that being initially BCR/ABL negative finally transformed into Ph-chromosome positive CML. In some of earlier papers that were published before Jak2V617F era there were a few mentions of BCR/ABL positivity in CMDs that was found by means of RT PCR. It gives us some reason to see if BCR/ABL positivity in CMDs still makes sense at least in the cases of poor clinical course and when the signs of progression and therapy resistance are visible. *Aim.* To collect blood samples from a number of CMD pts with unfavorable course and to see if some of them may demonstrate BCR/ABL positivity. *Methods.* To perform this study we have exploited qualitative and quantitative PCR detection of BCR/ABL fusion gene expression by means of RT PCR and RQ PCR. We have analyzed gene variants responsible for p190, p210 and p230 types of BCR/ABL protein synthesis. We have also performed detection of Jak2V617F, Jak2-ex12 and MPLW515L/K using AS PCR, direct PCR fragment sequencing and restriction analysis. This research was performed from 16.09.09 till 25.12.09. After receiving informed concerns blood samples (N=151) of CMD pts with unfavorable clinical course were collected in 8 local sites of Russia and sent to our lab. *Results.* We have examined 151 pts with CMD in progression (32(21%) - PV, 34(23%) - ET, 85 (56%) -IMF), median age was 62 y (23-86y), 98 females (65%), 53 males (35%). BCR/ABL expression was found in 43 of 151 (29%) CMD pts. Most of the cases were p210, only 2 were p190 and there were no p230. Jak2V617F was in 117 pts (78%), Jak2-ex12 was in 2 (1%), MPLW515L/K were not found; in 32 (21%) pts common Jak2 and MPL mutations were not found. BCR/ABL-positive (BA^+) and BCR/ABL-negative (BA-) groups show a distinct difference in leukocyte count: (L=9,4 (2,5-280)×10^9/L *vs.* 45(2,1-32,4)×10^9/L, P=0,017), other parameters were rather similar: Tr=439 (4,26-2000)×10^9/L *vs.* 485(93-5970)×10^9/L, Hb=133(62-218)g/L *vs.* 138(72-205)g/L, Er=4,3(2,49-9,3)×10^{12}/L *vs.* 4,9(2,4-7,3)×10^{12}/L. There was no difference in age and sex distribution among BA^+ and BA^-. The other prominent difference was hepatosplenomegaly (HSM): 40% in BA^+ *vs.* 28% in BA^-, P=0,021. BCR/ABL expression level in BA+ was rather low: 0,03(0,02-0,07)% IS (ELN International Scale). *Conclusion.* We have found a frequent (29%) BCR/ABL positivity detected by RT PCR and RQ PCR in CMD pts with clinical signs of the progression. This finding recalls the former discussion about the role of BCR/ABL in CMD pathology that seized down after Jak2 mutation era had begun. We suppose here that low BCR/ABL level in CMD may be a result of RNA editing rather than t(9;22).

1505

THE JAK2 RS10974944 SNP, PART OF JAK2 46/1 HAPLOTYPE, STRONGLY PREDISPOSES TO JAK2 V617F POSITIVE MYELOPROLIFERATIVE NEOPLASMS

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Background. Polycythemia vera, essential thrombocythemia and primary myelofibrosis are myeloproliferative neoplasms, characterized in a majority of cases by a unique somatic point mutation, JAK2 V617F. Recently, it was shown that JAK2 V617F occurs more frequently on a specific JAK2 haplotype, named JAK2 46/1. *Aims.* To evaluate the relationship between JAK2 rs10974944 SNP, part of the JAK2 46/1 haplotype, and JAK2 V617F mutation in a representative cohort of Romanian myeloproliferative neoplasms patients. *Methods.* We genotyped 149 myeloproliferative neoplasms patients (69 had polycythemia vera, 65 had essential thrombocythemia and 15 had primary myelofibrosis) with a known JAK2 V617F mutational status and 150 controls for the JAK2 rs10974944 (C/G) SNP (single nucleotide polymorphism), in which the G allele tags the 46/1 haplotype. *Results.* We found that the rs10974944 GG/CG genotypes were significantly enriched in patients compared to controls (P<0.0001). After stratifying for the JAK2 V617F mutational status and for the mutant allele burden, we demonstrated that GG/CG genotypes were significantly more frequent in V617F positive compared to V617F negative patients (P=0.001), but not in V617F negative patients compared to controls (P=0.29). Similarly, the GG/CG genotypes were significantly enriched in V617F positive patients with a mutant allele burden >50% compared to those with a mutant allele burden <50% (P=0.0006). *Summary/Conclusions.* Our results indicate that the G allele, part of the JAK2 46/1 haplotype, contributes significantly to the occurrence of JAK2 V617F - positive myeloproliferative neoplasms. Moreover, the JAK2 46/1 haplotype seems to be associated with mutant allele burden >50% in JAK2 V617F - positive myeloproliferative neoplasms patients.

1506

BIOPHISICAL PROPERTIES OF PLATELET MEMBRANE IN PATIENTS WITH PH-NEGATIVE CHRONIC MYELOPROLIFERATIVE DISORDERS

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Background. Patients with Chronic Myeloproliferative Neoplasms (MPN) often associate altered platelet function,these patients have more frequent thrombotic or haemorrhagic events.The purpose of the study is to identify the alterations of the platelet function in patients with MPN and to correlate changes in cell membrane fluidity with the platelet aggregation results. *Material and Methods.* This prospective study included 109 MPN patients (39 ET, 27 IMF, 43 PV) during the past 3 years in the Hematology Department . Platelet function was investigated by platelet aggregation with optical method and fluorescence anisotropy measurements. A group of 24 healthy volunteers was used for comparative *Results.* Statistics was performed using ANOVA. *Results and Discussion.* Platelets from patients with Ph negative MPN presented a low response to all the stimuli when compared to controls (ADP 30.97% *vs.* 70.25%, P<0.001; Collagen 27.2% *vs.* 70.86%, P<0.001; Epinephrin 10.68% *vs.* 73.33%, P<0.001; Ristocetin 28.76% *vs.* 71.7%, P<0.001).The low response of platelet aggregation is correlated with low fluidity of platelet membrane (ADP 0.1367for normal response *vs.* 0.1329 for low or absent response,collagen 0.1392 *vs.* 0.1310,epinephrine 0.1456 *vs.* 0.1232without statistical significance), ristocetin 0.1422 *vs.* 0.097-with statistical significance, P=0.02. Considering the type of MPN, we observed a statistically significant difference only for the amplitude of aggregation curves to ADP in ET patients *vs.* IMF patients (47.67% *vs.* 34.9%, P<0.05). The duration and the amplitude of the lag phase for ADP and collagen is higher for the patients with MPN *vs.* controls (ADP -amplitude 21.4% *vs.* 4.74%, P<0.05; duration 12.69% *vs.* 10.72%, P<0.05; Collagen -amplitude 23.84% *vs.* 4.74%, P<0.05; duration 94.69% *vs.* 10.72%, P<0.05). We observed a statistically significant correlation between the alterations of the lag phase and a higher medium platelet volume and thrombocrit (P<0.05). The incidence of deaggregation was higher for ADP (80/171 *vs.* 0/24,P=0.04), followed by ristocetin (22/162 *vs.*0/24) and collagen (15/70 *vs.* 0/0). Compared to the number of normal curves, the incidence of deaggregation to collagen is lower, but without a statistical significance. Patients with deaggregation have an high anisotropy than patients without deaggregation without statistical significance. Patients with MPN JAK positive presented major thrombotic accidents (portal vein thrombosis - 4cases, Budd-Chiari syndrome - 2cases, repetitive stroke - 3 cases). In these cases we observed a higher amplitude and slope to ristocetin *vs.* controls (83.1% *vs.* 71.7%), a lower incidence of deaggregation to ADP (2cases), collagen (no case), ristocetin (no case). The results are not statistically significant (not all the patients had access to detection of JAK mutation). *Conclusions.* The platelets of patients with Ph negative MPN presents more functional defects (altered platelet receptors, rigidity of membrane, insufficiency of granule release). The expression of platelet receptors are influenced by fluidity of platelet membrane. A low fluidity is correlate with low response especially for ristocetin. Presence of altered platelets determines variations of the lag phase and the possibility of deaggregation. JAK positive MPN may be associated with a higher incidence of major thrombotic accidents which may be correlated with a higher and a more rapid aggregation of platelets to ristocetin.

The informed consent was obtained.

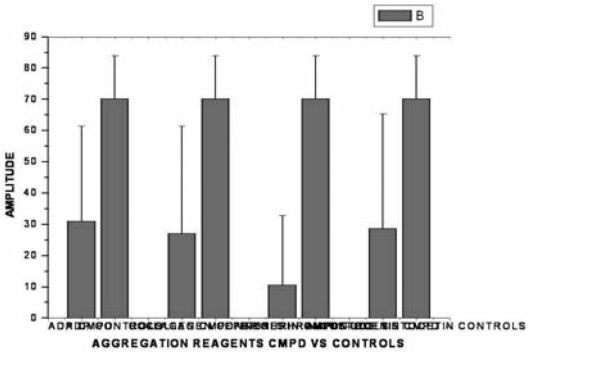

Figure. Platelet Aggregation MPN patients *vs.* controls.

1507

LEUKEMIA STEM CELLS (LSCS) INVADING LIVER IN THE MURINE MPD-LIKE MYELOID LEUKEMIA WITH LIVER LESION: MOLECULAR CHARACTERISTICS

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Background. LSCs are a small subset of leukemia cells capable for maintaining the bulk of tumor cells. Using a unique murine model of transplantable myeloid leukemia which gives metastases to the liver we were able to evaluate important characteristics of LSCs. After i.v. transplantation of 106 bone marrow or liver cells from affected mice the recipients die from acute liver failure due to massive invasion and proliferation of leukemic cells in the liver. The concentration of LSCs in the liver of moribund mice is very high and equals 1 LSC per 45 cells invaiding liver. *Aim.* The aim of this study was to characterize the properties of LSCs in given disease. *Methods.* Mice were injected with 106 bone marrow cell from leukemic donors. At the terminal stage of the disease $CD45^+$ cells were sorted by means of Miltenyi Biotec® MACS® magnetic separation columns from the liver of affected and control mice. RNA was isolated using guanidine isotyocianate. After evaluation of the quality of isolated RNA first strand cDNA synthesis was made using RT2 First strand kit (SABiosciences). The expression of 168 genes of interest was assessed by means of Real Time PCR using RT2 ProfilerTM PCR Arrays (SABiosciences). *Results.* Several known oncogenes were upregulated in CD45+ cells of leukemic origin compared to the same population of normal cells. Among them Runx1 (AML1), Etv6 (TEL), Cbfb, Vav1, Myc and components of AP-1 transcription factor c-Jun and c-Fos were elevated 47, 88, 53, 72, 26, 8 and 4 times respectively. Etv6 and Cbfb genes are common translocation parnters of Runx1 in different leukemias. Elevated expression of both points the potential first hit in studied leukemia. Transcription factors that are not oncogenes but regulators of hematopoiesis were also upregulated, for example Cepb-family genes Cebpb, Cebpe and Cebpg were overexpressed 80, 20 and 12 times respectively. It was shown that Notch signaling was activated in leukemic cells as Notch1 and Notch2 were overexpressed 13 and 72 times. It was shown that apparantly NF-kB pathway was activated in leukemic cells because activators (IL1), key regulators of this pathway (Ikbkb, Nfkbia) as well as many target genes (CyclinD1, Myc, Vcam1, Icam1, Nos2, cIAP1, cIAP2) were overexpressed. Housekeeping genes - Actb, Gapdh, Hsp90ab1, Hprt1 and Gusb were elevated 224, 221, 67, 48 and 38 times respectively and so were not suitable for normalization. Such high level of expression of these genes could be explained by active metabolism and rapid proliferation of leukemic cells. Nevertheless the expression level of several genes such as Fn1, Vegfa, Wnt1, Pax5, Jag1 and others did not change in leukemic cells and therefore could be used for normalization. *Conclusion.* Several oncogenes and transcription factors are overexpressed in analysed population of cells enriched with LSCs. This diversity could be explained by the accumulation of genetic events in constantly proliferating leukemic cells as well as the heterogeneity of studied population of cells. This model can be used for further characterizing LSCs, determing mechanisms of leukemic transformation and studying of extranodal involvement of malignant cells in leukemias.

1508

ENDOTELIAL CELLS PROTHROMBOTIC ALTERATIONS IN PATIENTS WITH MYELOPROLIFERATIVE DISORDERS AND BUDD-CHIARI SYNDROME: PRELIMINARY DATA

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Background. Endotelial cells (EC) are known to be involved by the malignant process in hematologic malignancies, such as chronic myelogenous leukemia and multiple myeloma. Recently, EC have been found to be interested also in patients with polycythemia vera (PV) and primary myelofibrosis (MF). Budd Chiari syndrome (BCS), due to thrombosis of the supra-hepatic veins, is a rare but dramatic complication in patients with PV or essential thrombocythemia (ET). It is more common in young females and in most of them it occurs with a mild increase in platelet count; sometimes other associated prothrombotic risk factors have been found. *Patients and methods.* In 4 patients with BCS (1 male with PV and 3 females with ET, mean age 35±3.2 years) we have performed biopsies of dorsal left foot vein to study the expression of CD31 (PECAM-1),CD34 and von Willebrand factor (VWF) on EC mem-

brane. EC were evaluated both with optical and electronic microscopy and histochemical studies were performed. *Results.* EC of our patients showed over expression of CD31 and CD34 on their membrane, as well as VWF. Electronic transmission microscopy showed in EC the presence of very high number of Weidel Palade bodies containing VWF. *Conclusions.* Vascular endothelium is known to provide a non-adhesive surface to circulating neutrophils and platelets while helping to prevent blood clotting. However, EC dysfunction might contribute to the hypercoagulable state associated with PV and ET. Recently, the presence of JAK2V617F mutation has been observed in EC of hepatic sinusoids belonging to patients with PV and BCS. We observed significant amounts of pro-thrombotic proteins in EC of peripheral veins of ET and PV patients and BCS. If these alterations are the result or the cause of the thrombotic event is not defined. The relationship between the EC involvement by the malignant process, the prothrombotic EC alterations and the development of thrombosis in ET and PV require further careful evaluation.

1509

MUTATIONAL ANALYSIS OF THE EGF RECEPTOR GENE IN BCR-ABL1 NEGATIVE AND JAK2V617F NEGATIVE CHRONIC MYELOPROLIFERATIVE NEOPLASMS

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Background. BCR-ABL1 negative chronic myeloproliferative neoplasms (CMPNs) are a heterogeneous group of clonal hematological malignancies. In last years, some genetic alterations have been described to cause these diseases, most of them activating tyrosine kinase (TK) genes. Tyrosine kinases proteins have an important role in cell growth and oncogenesis. Gain-of-function mutations in TK genes can produce a constitutive activation of several signaling pathways. *Aims.* In this study, we study the EGFR gene that codes for a tyrosine kinase receptor (RTK) involved in signaling pathways relevant in hematological cells. Mutations in this gene have been found involved in lung cancer, and it could have an important role in the pathogenesis of hematological disorders. *Methods.* We have analyzed the transmembrane and TK-coding domains of EGFR by dHPLC to detect mutations on samples from 44 BCR-ABL1 negative / V617FJAK2 negative CMPN patients and 20 control samples from healthy individuals. *Results.* Our results show that this gene is not frequently mutated in CMPNs. *Conclusion.* The EGF Receptor gene does not appear to be involved in the pathogenesis of the myeloproliferative neoplasms.

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1510

ENDOGENOUS THROMBIN POTENTIAL (ETP) IN MYELOPROLIFERATIVE DISORDERS (MPD)

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Background. People with MPD have an increased incidence of both arterial and venous thrombosis. Extensive coagulation studies have failed to derive a stable model of thrombosis in MPDs. In our study we associated thrombophilia in MPD patients measuring ETP parameters. *Aim.* Measurement of ETP parameters in MPD patients. *Methods.* The ETP parameters were studied in 24 patients with MPD [10 essential thrombocythaemia (ET 41.7%), 2 atypical MPDas (8.3%), 9 polycythaemia vera (PV 37.5%), 2 chronic myeloid leukaemia (CML 8.3%) and 1 mastocytosis (4.2%)]. 12 men (50%),12 women (50%) and a control group of 30 healthy subjects were recruited. We used the chromogenic method on the fully automated Behring Coagulation System (BCS) for the measurement of thrombin generation parameters. *Results.* Table. Patients with ET compared with patients with PV had decreased tlag (24 *vs.* 34.8), tmax (67.7 *vs.* 84.1) but increased Cmax (113.3 *vs.* 80) and ETP (409.1 *vs.* 312.2). The above results were statistically significant (P<0.008) except for tmax (P=0.211). ET patients had increased ETP compared to control group (409.1 *vs.* 394.7). *Conclusions.* Thrombotic tendency in MPDs, by ETP parameters, did not emerge in this study except the increase of ETP in ET compared to the control group. We must highlight the increased values of ETP in ET compared to PV. Prospective studies with larger numbers of patients are needed to assess the prognostic role of ETP values in thrombosis at MPD patients.

Table.

	Control group	MPD	P
tlag	19.3	29.3	0.0001
tmax	54.4	77.2	0.0001
Cmax	123.5	95.3	0.0001
ETP	394.7	358.1	0.033

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MONTHLY CYCLES OF ORAL MELPHALAN IN PATIENTS WITH PRIMARY AND SECONDARY MYELOFIBROSIS

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Background. Prolonged continuous oral Melphalan treatment was successfully employed in patients with Idiopathic Myelofibrosis (IM); however, a high incidence of evolution into blastic phase (BP) was observed. *Aim.* In order to reduce the incidence of BP and other side effects, a different Melphalan schedule with intermittent monthly cycles was tested at our Institution. *Methods.* From 1/2003 to 2/2009, 24 patients (16 males and 8 females, median age 66.2 years, range 41.7-76.2) were enrolled into the study; 19/24 had primary IM while in 5 there was a previous history of Polycythemia Vera/Essential Thrombocythemia. Six patients (25%) had been pretreated with Hydroxyurea. A symptomatic splenomegaly was present in all patients, with median spleen enlargement below costal margin of 12 cm (range 7-22). Median WBC level was 11.6×10^9/L (range 3.6-63.6), with 11/24 patients having WBC $>12.0\times10^9$/L; in addition, 3 patients had PLT level $> 500\times10^9$/L, 11 patients Hb level <10 g/dL and 3 patients had transfusional requirement. Melphalan was administered orally at the dose of 8 mg/day for 5 consecutive days every month. *Results.* Twelve patients (50%) had a reduction of spleen enlargement >75%, 2 patients >50% and 1 patient >25%, with a global response rate of 15/24 patients (62.5%): the remaining 9 patients had no variation in spleen volume. Elevated WBC values reduced to normal in all but one patient, elevated PLT values reduced to normal in 2 out of 3 patients, transfusional requirement disappeared in 1 out of 3 patients. Median number of cycles to best response was 10 (range 2-30). As concerns hematological toxicity, 4 patients had Hb decrease <8 g/dL, with 3/4 requiring transient transfusional support, and 1 patient had PLT decrease to $<50\times10^9$/L. One patient resistant to treatment died from broncopneumonia and 1 patient with normal PLT values died from cerebral hemorrhage; two patients had a grade 3-4 gastro-intestinal hemorrhage. Interestingly, only 2/24 patients (8.3%) had a BP evolution after 8 and 16 months from start of Melphalan, respectively. After a median number of treatment cycles of 18 (range 2-68), 12 patients are still in therapy and 12 discontinued Melphalan due to toxicity (4), resistance (3), relapse (3) or evolution to BP (2). *Conclusions.* The intermittent cyclic schedule of oral Melphalan seems capable to maintain a good response rate with reduction of symptomatic spleen enlargement in more than 50% of patients without excessive toxicity and with a lower rate of BP evolution, as compared to continuous Melphalan administration.

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BILINEAGE INVOLVMENT OF THE FUSION GENE IN A PATIENT WITH FIP1L1-PDGFRA POSITIVE CHRONIC EOSINOPHILIC LEUKEMIA AND T-CELL LYMPHOBLASTIC LYMPHOMA

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Background. There are successful attempts treating FIP1L1-PDGFRA positive chronic eosinophilic leukemia (CEL) with low dose imatinib mesylate. However, FIP1L1-PDGFRA fusion gene can induce both proliferation and differentiation of eosinophils, neutrophils, monocytes, lymphocytes and mast cells. A recent reports noted three cases with association of FIP1L1-PDGFRA positive CEL and T-cell lymphoblastic lymphoma (T-LBL). *Case.* We report on the case of 42 years-old man who presented with splenomegaly and lymphadenopathy. Peripheral blood count showed leucocytosis 51×10^9/L with marked eosinophilia 22×10^9/L and elevated promyelocytes and myelocites but no blasts. Bone marrow biopsy exhibited massive granulocyte hyperplasia with marked eosinophilia, iron deposits and 2nd grade reticular fibrosis. G-band karyotyping showed no abnormalities and BCR-ABL was absent in the peripheral blood. Histological examination of cervical lymph node biopsy detected T-LBL. 6 cycles of ACVBP chemotherapy was administered resulting in complete remission of T-LBL and decrease of eosinophil counts to upper normal range. However, eosinophilia progressed again to more 47×10^9/L in a couple of months after completion of chemotherapy. FIP1L1-PDGFRA transcripts by RT-PCR were identified in peripheral blood, bone marrow and the lymph nodes affected by T-LBL. The final diagnosis of FIP1L1-PDGFRA positive CEL and T-LBL was established. Blood and bone marrow counts normalized after the initiation of imatinib mesylate 100 mg/d. 2 years after the final diagnosis the patient is alive in complete clinical, hematological and molecular remission on imatinib 100 mg/d. *Conclusion.* FIP1L1-PDGFRA-positive CEL in conjunction with T-cell LBL suggests a bilineage cell involvement probably arising from an early hematopoietic progenitor. Identification of FIP1L1-PDGFRA in cases of T-LBL with concurrent eosinophilia helps to select the targeted tyrosine kinase therapy.

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THE ASSOCIATION OF JAK 2 V617F MUTATION AND LEUKOCYTOSIS WITH THROMBOCYTIC EVENTS IN ESSENTIAL THROMBOCYTHEMIA

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Background. Essential thrombocythemia (ET) is a chronic myeloproliferative disorder that primarily involves the megakaryocytic lineage, and is characterized by sustained thrombocytosis in the blood and increased number of large, mature megakaryocytes in the bone marrow with the sensitivity to several different growth factors. A recently discovered mutation in the gene for Janus kinase 2 (JAK 2 V617F - exon 14 mutation) on chromosome 9p 24 offers and explanation for this hypersensivity. The JAK 2 V617F mutation is found in approximately 50% of the ET patients predominantly in the heterozygous form, while 42% of the patients have wild type. We investigated the frequency of mutations in ET patients and analyzed the relationship with their clinical features. *Material and methods.* Eighty-four ET patients were evaluated in our study. The amplification refractory mutation system was applied for the mutation survey of JAK2 V617F. DNA was extracted from whole blood samples with NucleoSpin (Macherey-Nagel) kit according to manufacture's directions. Analysis was performed using the JAK2 MutaScreen kit for detection of JAK2 V617F mutation (Ipsogen) Real-Time PCR by LightCycler 2.0 instrument. *Results.* Fifty-three (64%) patients harboring the JAK2 (V617F) mutation, including 4 homozygous and 49 heterozygous changes during follow-up, 27 (32%) patients suffered from documented thrombotic events, with 24 having JAK2 V617F mutations. Statistical analysis showed that patients with the JAK2 mutation had significantly higher hemoglobin level, leukocytes, and thrombotic events (P=0.027, P=0.01, and P=0.024), respectively. Thrombotic events were also significantly correlated with age above 60 years, previous history of thrombosis and leukocytosis. No correlations were found between long duration of thombocytosis, number of platelets and JAK2 V617F. *Conclusions.*Our findings supports the opinion that both factors, JAK2 V617F and leukocytosis should be taken into account when ET patients are evaluated for risk of thrombotic complications. Therefore, detection of the JAK2 V617F mutation can affect not only the diagnosis, but also the management of ET patients.

1514

CHRONIC EOSINOPHILIC LEUKAEMIA - NOT OTHERWISE SPECIFIED (CEL-NOS) A POOR PROGNOSED DISORDER WITH RESISTANCE TO CONVENTIONAL CHEMOTHERAPY

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Background. Chronic eosinophilic leukaemia (CEL) is a myeloproliferative neoplasm characterized by clonal proliferation of eosinophil precursors resulting in increased eosinophilia count in peripheral blood and marrow with subsequent organ damage. Material and *Methods.* Ten patients (7 male/ 3 female) with a median age of 64 years (range 23-77) were diagnosed as having CEL according to criteria proposed by WHO classification 2008. Patients who met the criteria for idiopathic hypereosinophilic syndromes and CEL with FIP1L1-PDGFRA transcripts were excluded. *Results.* Eight patients had increased number of blast cells in peripheral blood and/or bone marrow or the presence of cytogenetic abnormality, in the remaining 2 cases- JAK2V617F point mutation was documented. The organ involvements included splenomegaly (n=6), hepatomegaly (n=4), mitral valve regurgitation (n=1), cardiac failure (n=1), peripheral neuropathy (n=2). A median white blood cell (WBC) count was 23.9×10^9/L (range 9.3-175) with absolute eosinophil count (AEC) of 9.6×10^9/L (1.5-136) and eosinophil marrow infiltration of 37% (14-64). Serum IgE and vitamin B12 levels were 151 Iu/mL (3.8-856) and 813 pg/mL (410-3359) respectively. The cytogenetic exam was available in 8 patients and revealed normal karyotype in 5, -4 in 1, -11,-19 in 1 and -7 in 1. A median number of treatment lines was 3 (1-5). All patients were resistant to conventionally used agents such hydroxyurea, busulphan, imatinib, interferon and low dose cytarabine. One patient achieved complete remission after allogeneic stem cell transplantation. Two patients remain in partial response while on hydroxyurea with prednisone. Seven patients died from 1) progression to acute myeloid leukaemia (n=3), 2) concurrent development of lymphoblastic lymphoma (n=1), 3) cardiac complications in refractory CEL (n=3) and 4) myocardial infarction (n=1). *Conclusions.* CEL-NOS is a rare disorder with resistance to conventional chemotherapy. Allogeneic stem cell transplantation remains the only curative therapeutic option.

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ANAGRELIDE TREATMENT AND PROSPECTIVE CADIOVASCULAR EVALUATION IN ESSENTIAL THROMBOCYTHAEMIA PATIENTS: A PILOT STUDY OF THE REGISTRO ITALIANO TROMBOCITEMIA (RIT)

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Background. The cardiovascular (CV) adverse events are reported in the Essential Thrombocythaemia (ET) patients treated with Anagrelide as a cause of treatment withdrawal. *Objective.* To define the role of an attentive CV monitoring in the ET patients candidate to or receiving Anagrelide treatment. *Materials and methods.* Twenty-nine ET patients (PVSG or WHO criteria) potentially candidate to Anagrelide treatment were object of this observational prospective study. The planned CV evaluation included clinical aspects as family and personal history, general CV risk factors, physical examination; biological parameters as Troponin I and Pro-BNP; instrumental parameters obtained with ECG, Echocardiography, and if indicated with Holter ECG and ergometric test. This CV evaluation was planned to be done before Anagrelide treatment and then after 3-6, 9-12, and 18-24 months. *Results.* All 29 patients were judged able to be treated with Anagrelide, but only 18 of them actually started the treatment and are object of this report. The patients, 11 females and 7 males, aged 14-67 years (median 42), had at diagnosis a median PLT count of 756×10^9/L (range 456-1518). The JAK2 V617F mutation was documented in 8 cases (44 %). At the start of Anagrelide treatment all patients had previously received a cytoreductive therapy, and were receiving Aspirin 100 mg/day. The Anagrelide dose at start was 1 mg/day and during the maintenance phase had a median

value of 1.5 mg/day (range 1-3 mg). The baseline CV evaluation documented no abnormalities contraindicating the treatment with Anagrelide (NYHA Class I in 18/18 cases). A palpitation was reported in two cases, and the heart rate was >100/min in one case, being the median value 81; the ECG was normal in 18/18; the Echocardiography documented: Ejection Fraction (EF) >50% in all 18 cases with a median value 66%; a left ventricle hypertrophy in two cases; and a minor valvulopathy in six cases. Supplementary information were obtained in four patients with ergometric test and Holter ECG. The Troponin I was always normal and the Pro-BNP was elevated in one case. During the follow-up, after 3-6 and 9-12 months in 12 cases and after 18-24 months in 6 cases, the median PLT count was 461, 355, and 210×10^9/L, respectively; the median pulse rate was 80, 77, and 75/min; the median value of EF was 68%, 66% and 65% (always >50%); the ECG, Troponin I and Pro-BNP were always normal; a palpitation occurred in two cases; a palpitation associated with arterial hypertension in another case was cancelled with use of a beta-blocker; Oedema occurred in two cases. The Anagrelide withdrawal was due to the occurrence of grade III-IV oedema in one case, and to the planning of pregnancy and of a BMT procedure in other two cases. *Conclusion.* This pilot prospective study shows that an attentive CV monitoring may be very useful to safely enrol and treat ET patients with Anagrelide.

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DETECTION OF MUTATIONS ASSOCIATED WITH CHRONIC MYELOPROLIFERATIVE DISORDERS USING BEAD-BASED LIQUID ASSAY

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Background. The JAK2 V617F mutation has recently been reported to be associated with the pathogenesis of chronic myeloproliferative disorders (CMPDs). Several techniques such as restriction fragment length polymorphism, direct sequencing analysis, pyrosequencing, real-time AS-PCR and a denaturing high-performance liquid chromatography have been developed for the detection of this kind of mutation. *Aims.* We aimed at the establishment and validation of a novel rapid, cost-effective and moderate-throughput method for JAK2 V617F mutation identification using a multiplexed bead-based suspension array platform - LuminexRxMAPTM. *Methods.* A 354 bp region of spanning exon 14 of the human JAK2 gene was amplified using biotinylated primers and genotyping was performed by direct hybridization with 2 oligonucleotide probes, specific for the wild type and mutant alleles. The probes were synthesized with 5' amino group and 20 bases spacer oligonucleotides to allow covalent binding to carboxylated microbeads (Luminex Corp). *Results.* The method was validated by testing on different proportion mixtures of artificial plasmid construct harboring either the wild type and mutant JAK2 allele and a panel of DNA samples from patients with known CMPDs, genotyped by direct DNA sequencing. The sensitivity of this novel assay was approximately 5% mutant DNA in a wild-type *Background.* No discrepancies between the novel Luninex-based method and sequencing were observed. *Conclusions.* Our novel method could be successfully implemented in the diagnostic work-up for CMPDs. Additionally, the assay allows quantification of the JAK2 mutant allele burden and therefore is applicable for assessment of minimal residual disease in patients undergoing JAK2 targeted therapy or alloHSCT. Furthermore, this system would allow the design of multiplex assays for simultaneous testing for the presence of various mutations associated with CMPDs. *Acknowledgements.* This work was supported by Grant ID_09_157 (Contract 5/16.12.2009) of the National Science Fund, Bulgaria. Research activities of *vs.* and EH are also partially supported by Grant CV_119_09 of the National Science Fund, Bulgaria.

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A NEW JAK2 EXON12 DELETION IN AN ELDERLY WOMAN WITH POLYCYTHEMIA VERA

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Background. Mutations in the JAK2 gene, mainly the JAK2V617F, are present in about 95% of the Polycythemia Vera (PV) patients. Since 2007, mutations in the exon 12 have been described in a minority of patients previously described as PV JAK2V617F negative or idiopathic erythrocytosis (IE). Apparently, patients with JAK2 exon12 mutations have higher haemoglobin levels than the other PV patients, normal leukocytes and platelets counts and isolated bone marrow erythroid hyperplasia. The prevalence seams to be higher in females and younger age patients comparing with JAK2V617F positive PV. *Aim.* To describe the clinical case of a female presenting at the age of 87 with Polycythemia Vera and a JAK2 exon12 mutation not previously described. *Methods.* Mutation studies were performed in genomic DNA extracted from peripheral blood leucocytes and bone marrow. JAK2V617F was screened by ASO-PCR. JAK2 exon 12 mutations were investigated by direct sequencing. *Clinical Case.* In November 2009 a 87 years old female was referred to our Hematology out-patient clinic to investigate an erythrocytosis accidentally discovered in pre-surgery routine analysis (uterine prolapse). Hb 20 g/dL, Ht 70%, WBC 10.4×10^9/L, Plat 353×10^9/L. Patient complains with headache, dizziness, facial plethora and exercise dyspnea for the last 2 years. She is non smoker, with controlled arterial hypertension, auricular flutter (NYHA 2) and a previous surgery for uterine prolapse (2002). She is not aware of other family member with blood disorders. Non haematological conditions causing erythrocytosis were excluded. The level of EPO was 7,6 mUI/mL (normal 3-30) and Ferritin 10,7 ng/mL (normal 9-120). Molecular studies: Screening for JAK2V617F was negative. Direct sequencing of JAK2 exon12 revealed the mutation R541-D544del in the DNA extracted from peripheral blood leukocytes and bone marrow. Cytoreductive treatment with Hydroxiurea was initiated. *Conclusions.* This new JAK2 exon12 deletion R541-D544 occurs in a highly specific cluster of activating mutations occurring between residues 537 and 543 (Butcher *et al.*). We previously described a 17 year old male with splenomegaly, splenic vein thrombosis, Hb 23,7g/dL, Hct>60%, normal leukocytes and platelets counts, with a JAK2 exon12 deletion in the same region, R541-E543delinsK. This new JAK2 exon12 R541-D544del mutation was discovered in an elderly woman whose most relevant anomaly was an isolated erythrocytosis.

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MYELOID NEOPLASM WITH FIP1L1-PDGFRA REARRANGEMENT RESPONSIVE TO IMATINIB DESPITE LONG TERM EVOLUTION

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Background. Myeloid and lymphoid neoplasms with eosinophilia and rearrangements of PDGFRa, PDGFRb or FGFR1 have been recognised a separate category in the 2008 WHO classification of tumours, most common being PDGFRa rearrangements. Main features recognised to date are eosinophilia, splenomegaly, cardiac involvement, thrombosis and elevated serum levels of cobalamin and tryptase. Commonly the PDGFRa rearrangement can only be detected by PCR or FISH; cytogenetic analysis is normal and additional abnormalities (trisomy 8) are likely to represent disease evolution. This disease carried a dismal prognosis before an exquisite response to imatinib was reported, so reports on long term evolution are scarce. We report a case of a 55 year old male with eosinophilia and PDGFRa rearrangement diagnosed in evolution to acute leukemia after 13 years' evolution. *Case report.* The patient was first studied in 1996 because of persistent eosinophilia above 1500 per cubic milimeter. After a steady increase in eosinophils, a bone marrow examination was performed in 2003 yielding normal counts and cytogenetic analysis; a diagnosis of idiopathic hypereosinophilic syndrome was made and the patient remained asymptomatic and therefore untreated for six more years. In 2009, the patient was admitted to hospital with persistent fever, night sweats, fatigue and anorexia. Splenomegaly was the only remarkable physical finding, confirmed by CT scan to be 20 cm in size. Blood counts showed normal hemoglobin and platelets, along with leukocytosis of 61000 per cubic milimeter. Most remarkable findings in differential count were eosinophilia of 22000 per cubic milimeter, left shift and myeloblasts accounting for 3% of leukocytes. Serum chemistry yielded a LDH of 1105 U/L,normal tryptase and cobalamin of 1610 pg/mL. Echocardiography found a normal heart structure and function. Bone marrow counts confirmed considerable eosinophilia and 11% myeloblasts; no excess mast cells were noted. Trisomy 8 was the only finding on conventional marrow cyto-

genetics, confirmed by FISH in 35% nuclei; FISH also detected FIP1L1-PDGFRa rearrangement in 90% cells. Imatinib therapy was initiated and an hematologic response reached within two weeks, preceded by mild cytopenias not requiring drug withdrawal. Trisomy 8 and FIP1L1-PDGFRa rearrangement were undetectable by FISH in peripheral blood in four weeks. From then on, blood counts are within normal limits and the drug is administered at 100 mg three times a week. Comments on this case: Natural history of eosinophilia with PDGFRa rearrangement can span several years before symptoms arise. Initial imatinib response is maintained despite long term disease evolution and clonal evolution. Trisomy 8 is considered part of the malignant clone, because of response to imatinib therapy.

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SYSTEMIC MASTOCYTOSIS: AN-UNDER DIAGNOSED DISEASE. A COHORT STUDY WITH 12 CASES

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Background. mastocytosis is a neoplastic disease characterized by malignant transformation of mast cells (MC), and which symptoms are a consequence of proliferation, accumulation of the mast cells, as well as liberation of biological mediators. There are several diagnostics criteria. *Major Criteria.* Multifocal dense mast cell (MC) infiltrates (≥15 MC/infiltrate) in the bone marrow or in any other internal organ. Minor Criteria: 1) >25% spindle-shaped cells in MC-infiltrates; or >25% of all MC are atypical MC (type I and/or type II) in bone marrow smears; 2) expression of CD2 and/or CD25 on bone marrow MC3; 3) serum tryptase level >20 ng/mL; 4) c-kit point mutation at codon 816 (mostly D816V) in bone marrow or in another internal organ. The diagnosis of the Systemic Mastocytosis is established if at least 1major and 1 minor, or 3 minor criteria are fulfilled. The clinical courses of mastocytosis vary. The majority of the patients, especially those with Cutaneous Mastocytosis (CM) or with Indolent Systemic Mastocytosis (ISM), demonstrate an indolent period that could last for many years or even decades, while other patients, such as with Systemic Aggressive Mastocytosis (SAM) or Systemic Mastocytosis Associated with Hematological Malignant Disease (SM-AHMD), present with an aggressive course with frequent lethality. *Aims.* Retrospective study aimed at description and analysis of clinical data of the patients with mastocytosis of the Hematology Department. We have analyzed clinical cases between 1998 and 2009. We have revised the following results of the analysis: previous clinical symptoms, data from the blood tests, calcium metabolism, tryptase and histaminuria, hormonal assays, immunophenotype of the bone marrow samples, mutational analysis of the Asp816/Val (D816V) and cKit in neutrophils/monocytes/eosinophils. *Methods and Results.* Among the 12 studied patients, 7 were males and 5 females, with ages between 4 and 79 years. Two of them presented with CM, six with ISM, and three patients had SM-AHMD. Patient with ISM showed from 0.01% to 3% of mastocyte infiltration in bone marrow. The serum tryptase levels varied between 6.6 mcg/L and 189 mcg/ç. The D816V mutation was detected in 7 cases, while two cases were negative. In one patient we detected a different mutation not clasificable and in two of the patients the mutational analysis was not possible to do. All the patients had similar general symptoms, including head ache, itchy skin, flushing, hypotension, abdominal pain with gastrointestinal symptoms. In eight cases we observed bone pathology, including two cases with osteosclerosis. The four cases with SAM presented hipoalbuminemia, organomegaly, splenomegaly and three of them hematological alterations. All the of these patients received symptomatic treatment, including disodic chromoglycane, antihistaminics H1 and H2, and steroids. Four patients were treated with interferon with good clinical response in only one case without hematologycal alterations. *Conclusions.* we believe that the systemic mastocytosis is an under-diagnosed disease because patients are treated for years by different specialists, who partially relieve symptoms without final diagnosis and adequate treatment. We conclude that it is important to diagnose early and start early adequate treatment, especially in cases SM-AHMD, which have poor prognosis.

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THE IMPORTANCE OF SCORING SYSTEM DEFINING IN THE PROGNOSIS OF PATIENTS WITH PRIMARY MYELOFIBROSIS

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Background. Primary myelofibrosis (PMF) is a clonal myeloproliferative neoplasm (MPN) characterised by a proliferation of predominantly megakaryocytes and granulocytes in the bone marrow that in fully developed disease is associated with reactive fibrosis and with extramedullary haematopoiesis. *Aim.* Define initial, clinical, haematological and pathohistological features of patients with PMF, as well as classify patients into the risk groups according to different scoring systems (Lylsk, Kelnsk, Tefferi, Meyo, IPSS, Strasser, Cervantes) and assess the prognosis importance of previously mentioned parameters and scoring systems. *Patients and methods.* 77 patients with PMF, F/M 32/45; at the average age of 59.3 yrs (29-79 yrs) were examined and treated at the Institute of Haematology in the period from November 1995 to January 2006. Primary clinical, haematological and pathohistological parameters of the disease were determined with each of the patients. The degree of hepatosplenomegaly was expressed in cm palpability below costal margin. Dying according to Gomori was used for reticular fibrosis as well as Azan according to Heidenheimu for collagen fibrosis. Cytogenetic analysis was performed applying HG-banding technique. The patients were divided into three risk groups according to the following scoring system: Lylsk, Kelnsk, Tefferi, Strasser, IPSS, Meyo and Cervantes. Statistical *Methods.* descriptive statistics, graphical displaying, and Kaplan-Meier for the surviving probability. *Results.* Average time of patient surviving was 82.8 months in the range of 9-120 months, whereby 24 patients died. Statistically important factors of shorter surviving period were: the male sex, Hb<100g/L; Platelets <100×10^9/L; prominent leucoerythroblastosis with dacryocytosis; % of myeloblasts >2%; advantage stage of disease, hepatomegaly, and presence of constitutional symptoms. Starasser, Cervantes and Kelnsky's scores didn't display any statistical importance concerning the period of surviving. Lylsk, Tefferi, Mejo and IPSS scoring system pointed out significant differences of the length of surviving period between low risk and intermediate risk groups (P<0.05), but without any importance between high risk and intermediate risk groups (P>0.05). *Conclusion.* The choice of treatment options and surviving of PMF patients are still unsatisfactory. Prognosis scoring systems have great importance in the choice of optimal treatment and the time of its initiating.

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FAVORABLE RESPONSE TO ALPHA-INTERFERON IN A PEDIATRIC PATIENT WITH AGNOGENIC MYELOID METAPLASIA COMBINED WITH FV LEIDEN ANOMALY

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Background. Agnogenic Myeloid Metaplasia with myelofibrosis (AMM) is a myeloproliferative disease chracterized by leukoerythroblastosis, myeloid metaplasia and variyng degrees of myelofibrosis with very low incidence in pediatric patients. Symptoms inlude malaise, weight loss and splenomegaly. AMM is clonal disease but the JAK2V617F mutation seems to be less frequent than in adults. The natural history can be variable with survival variyng from 1.5 to 5 years. The treatment is ill defined although hydroxyureea, anagrelide and alpha-Interferon have been used. *Aim.* we present a case of a 17 years old girl diagnosed in March 2009 with AMM and thrombophilia (FV Leiden anomaly) with wery good response to alpha-Interferon (alphaIFN). *Methods.* diagnosis was formulated after clinical examination, peripheral blood analysis, bone marrow biopsy, cellular colonies assays and molecular tests for BCR/ABL, JAK2 and coagulation FV Leiden mutation. *Results.* the onset consisted in weight loss, subfebrility, upper abdominal pain with nauseea and emesis. The clinical examination showed palor and large hepato-splenomegaly. Initial blood count revealed Hb= 10.5 g/dL, WBC= 6.8×10^9/L, PLT= 919×10^9/L, and blood

smear examination showed marked anisopoikylocytosis, dacryocytosis, leukoerythroblastic picture. Bone marrow aspirate was hipocellular. Bone marrow biopsy showed hiperplasia with panmyelosis, hiperplasia of atypical megakaryocytes (with nuclear hiperlobulation, disposed in perivascular and paratrabecular large areas); G/E = 3/1; diffuse mild densification of reticuline network. Molecular testing for BCR/ABL and JAK2 mutation were negative. The colony assay showed incresed numbers of GM-CFU and the presence of erythroid endogenous colonies with slowly increased level of seric erythropoietin level. The abdominal ultrasonografic examination, CT-Scan and MRI revealed marked hepato-splenomegaly, portal cavernoma and extensive thrombosis of portal, splenic, suprahepatic veins. The superior endoscopy showed the presence of esophageal grade I varices. Hemostasis evaluation showed global alteration of coagulation screening tests (prolonged PT, APTT), normal C ans S porteins, with abnormal activated protein C resistence. The PCR test for FV showed the presence of FV Leiden mutation in heterozigot form. HLA-typing identified a full-match donor among her brothers. The patient was initially treated with alpha IFN (3 MU / 3 times per week), low dose aspirin (75 mg/day) and propranolol (40 mg/day) in order to decrease the portal hipertension. The patient is followed on a monthly basis with blood counts, coagulation tests and atevery 3 month with abdominal ultrasound. After the treatment we obtained a very good response consisting in remission of hepatosplenomegaly and normalization of platelets counts after 10 weeks with maintainace of normal clinical and hematological picture at 1 year after diagnosis. *Summary.* AMM is a very rare disease in children and adolescents, with few cases reported in the literature making it difficult to establish a standardized treatment. The favorable response of this case after treatment with alpha IFN led us to postpone the indication of sibling allogeneic stem cell transplantation.

1522

PRIMARY MYELOFIBROSIS: FREQUENCY OF GENETIC ABNORMALITIES IN BRAZILIAN PATIENTS AND INCREASED FREQUENCY OF PATIENTS YOUNGER THEN 50 YRS

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Primary myelofibrosis (PMF) is a myeloproliferative neoplasia characterized by progressive marrow (BM) fibrosis, extramedullary hematopoiesis with splenomegaly, anemia and peripheral blood (PB) smear showing teardrop red cells and leukoerythroblastosis. Around 50% of cases present JAK2 V617F, while MPL W515L/K mutations are present in <9% of patients without JAK2 V617F. The aim of this study was to describe the frequency of cytogenetics aberrations (karyotype and FISH), detect JAK2 and MPL W515K/L mutations and classify a group of PMF patients in a prognostic scoring system. Twenty patients with PMF consecutively diagnosed according to WHO classification of tumors at Unifesp, São Paulo, Brazil, were selected for the study. Diagnose work up included: PB and BM cell morphology analysis, BM biopsy, karyotype, FISH, JAK2 and MPL mutations detection among others tests. Male to female ratio was 0.8:1; median age was 65 years (from 42 to 88), three patients (15%) were younger then 50 years-old; median spleen size was 7cm from LCM (from 0 to 17cm); mean Hb level was 11.2g/dL (from 6.4 to 16g/dL), anemia was present in 13 (65%) patients; mean WBC was 16132/μL (from 3680 to 84000/μL); WBC exceeded 10000 in 13 (65%) cases; only one patient presented leucopenia; mean platelet count was 660450/μL (from 2000 to 1600000 /μL); thrombocytopenia was found in three (15%) patients and thrombocytosis in 11(55%). Karyotype was successful in 14 patients, and three (22%) of them were abnormal: two cases with +8 and one +9. FISH using probes for centromere of 8, centromere of 9, del(13q) and del(20q) were performed in cases with normal karyotype or unsuccessful results but did not reveal additional abnormalities. JAK2 V617F was detected in 11 (55%) patients and MPL W515K/L was detected in one (5%) case without JAK2 V617F. According to a scoring system based on two adverse prognostic factors, namely Hb below 10g/dL and WBC below 4000/μL or greater then 30000/μL (Dupriez *et al.* 1996), 14 patients were classified as low risk and 6 as intermediate risk. According to independent cytogenetic risk categorization (Hussein *et al.*, 2010), patients with +8 had an unfavorable prognosis with 34m of survival as compared to +9 with 113m. In fact, one of the +8-patients who was classified as intermediate risk was the only death observed in this group in the 5-year-follow up period, confirming literature data. The patient with MPL W515K/L mutation was an 87yr-old female with severe anemia (6.4g/dL). As described, PMF associated with MPL mutations tends to commit more frequently older women, and leads to a more intense degree of anemia. In conclusion there was an increased frequency of PMF (15%) in younger patients (<50yrs), phenomenum also observed in CML, a disease of youth in Brazil (de Campos *et al.*, 2010). The frequency of JAK2 as well as MPL mutations was similar to international series; the frequency of chromosomal abnormalities was a bit smaller and +8 stratified unfavorable evolution.

1523

LEUKEMIC TRANSFORMATION OF PHILADELPHIA CHROMOSOME-NEGATIVE MYELOPROLIFERATIVE DISORDERS: ARE ASIAN PATIENTS DIFFERENT?

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Background. Leukemic transformation (LT) is rare in the natural history of Philadelphia (Ph) chromosome-negative myeloproliferative disorders (MPD). Prognosis is dismal with a median survival of less than 5 months irrespective of chemotherapy regimens, type, duration and treatment of MPD. Little literature is available on Asian patients. *Aims.* To study LT of Ph chromosome-negative MPD in Asian patients and determine the disease profile in Asian population. *Methods.* Patients were recruited from the MPD Registry of Singapore General Hospital Department of Hematology, an Ethics Committee (EC)-approved computerized database capturing clinical data of patients diagnosed with Ph chromosome-negative MPD from 1980 to 2009. Patient informed consent was waived by EC. Ph chromosome-negative MPD were diagnosed based on accepted criteria in use at time of diagnosis. All LT cases fulfilled WHO criteria and were included in the study. Reviews of clinical data including MPD diagnosis, treatment modalities and duration of use in myeloproliferative phase, latency to LT, type and characteristics of leukemia, chemotherapy administered and survival after LT were examined. *Results.* Over the 29-year period, there were 22 Asian patients with LT of Ph chromosome-negative MPD. LT incidence is 3% (22/725). Four of the patients had polycythemia rubra vera (PRV), 9 essential thrombocythemia (ET), 7 myelofibrosis (MF) and 2 MPD unspecified (MPD-U). 46% were males. 95%(n=21) were Chinese and the other patient was Malay. Median age at LT diagnosis was 67.5 years. All 4 PRV patients had received hydroxyurea(HU) for a mean duration of 10.5 years before LT. Eight of 9 ET patients had received HU for mean duration of 8.8 years. One had 32P, an alkylating agent. Five of 7 MF patients received HU, for a mean duration of 1.6 years. One MPD-U patient had HU for 1 year and the other presented with acute leukemia with marrow features of MPD. Median latency to LT was 14 years for PRV, 10 years for ET and 1 year for MF. Nineteen patients had myeloid leukemia, 2 were biphenotypic leukemia and 1 patient presented with granulocytic sarcoma. Nine patients had complex cytogenetics, with abnormalities of chromosomes 5 and 7 being common. Overall prognosis of patients who developed LT was dismal. Eight patients received AML induction chemotherapy but failed to achieve durable remissions. Fourteen patients were given low-dose chemotherapy for palliation or supportive care because of advanced age or poor performance status. None of the 22 patients received transplant because of refractory disease and /or overwhelming infections. Median survival of patients was 2 months. Survival was independent of MPD type and treatment administered. *Conclusion.* LT of Ph chromosome-negative MPD is rare and uniformly fatal. HU was the primary treatment for most MPD but it is difficult to determine its leukemogenecity from this cohort. Transformation was mainly to myeloid leukemia. Common cytogenetic abnormalities involved chromosomes 5 and 7. Despite chemotherapy, survival was poor and patients succumbed to refractory disease and infections. Asian patients did not have a more favorable outcome. It remains to be investigated whether upfront stem cell transplant may be a treatment option.

1524

INTERSTITIAL PNEUMONITIS ASSOCIATED WITH ANAGRELIDE IN A PATIENT WITH ESSENTIAL THROMBOCYTHEMIA

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Background. Anagrelide (ANA) is a quinazoline derivative that acts selectively on megakaryocite proliferation and maturation. In cases of Essential Thrombocythemia (ET) ANA is indicated in young patients of high risk who need to initiate cyto-reductive therapy and in patients with clinical resistance or intolerance to hydroxyurea. ANA-related

adverse events are infrecuently dyspnea and pneumonia, and rarely pulmonary infiltrates. We have only found two cases of Interstitial Pneumonitis in the literature related to ANA therapy. *Case summary.* A 54-year-old woman was diagnosed with ET in May, 2002. She was offered Anagrelide to control thrombocytosis (baseline platelets > 1.500×10^9/L) after cardiological evaluation. After one month she achieved normalization of the platelet count (platelets 223×10^9/L). She needed 3 mg/día of ANA and showed a good tolerance. Two months after initiating the treatment, she was admitted to the Hospital with a progressive difficulty in breathing in case of moderate effort, with cough, costal pain, and a feverish sensation of 4 days evolution. She presented the following results from exploration: temperature 38ºC, AT 100/50, tachypnoea, bilateral fine crackles at auscultation. Blood test: leukocytes 8.1×10^9/L, Hb 10.7g/dL, platelets 129×10^9/L. The pulse oximeter reading was 90%. Blood gas: pH 7.49, pO2 55, pCO2 27. Chest-X-rays: bilateral alveolointerstitial infiltrates with bilateral pleural effusions. CT of the chest showed patchy areas of ground-glass opacifications that mainly involve the upper lung zones and small alveolar peripheral infiltrates in lower right lung zone. Echocardiogram was normal. We discard infection for Legionella and Micoplasma. Pneumonitis for hypersensitivity by ANA was suspected so the medication was suspended. There is a clinical, functional and radiological progressive improvement, it not being necessary to establish corticosteroid therapy. In ambulatory control the patient does not have dyspnea, with platelet count of 1.000×10^9/L. Pulse oximeter and Chest-X-ray are normal. The case is notified to Andalusian Pharmacovigilance Centre. According to the algorithm of causality of Karch-Lasagna modified, it qualifies as "possibly", coinciding with the category assigned by the Centre. The reaction is considered to be serious. *Conclusions.* In all the patients who take ANA it is necessary to monitor both the cardiac and the pulmonary function. Vigilance is advised in patients who develop cough or dyspnea to maintain awareness of the possibility of the development of Pneumonitis.

1525

IS ORGAN TRANSPLANTATION CONTRAINDICATED DURING POLYCYTHEMIA VERA?

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Background. Chronic myeloproliferative neoplasms are characterized by long lasting evolution complicated by vascular complications and haematological transformation (secondary myelofibrosis, acute leukaemia). Risk factors for these complications are bad understood. It is generally admitted that clonal haematological disease represent contraindication for organ transplantation. Consequences of transplantation-induced immunodepression on evolution of chronic myeloproliferative disorder are bad described, in particular on the risk of aggravation or haematological transformation. *Aims.* We report the case of a man with polycythemia vera who has benefited from a renal transplantation. *Methods.* A 39 year-old man was diagnosed with polycythemia vera. He was treated with phlebotomies and then hydroxyurea with good response. He suffered also from a renal polykystosis and developed progressively a renal insufficiency with need from dialysis. After 2 years, it was decided to perform a renal transplantation. A complete evaluation with bone marrow biopsy excluded progression to secondary myelofibrosis. Jak2 mutation was positive before transplant at 75%. This procedure allowed us to monitor evolution after renal transplantation. After renal transplantation, hyperleucocytosis was noted and necessitated to augment hydroxyurea dose. After 18 months of follow up after renal transplantation, the patient was still in good haematological response on hydroxyurea. No significant modification of Jak2 allele burden was noted. *Results.* Indication for organ transplantation in the context of clonal haematological disease is always difficult to retain, especially in chronic disorder. Literature data are scarce. Case reports suggested that immunodepression can deteriorate haematological disease with even transformation in acute leukaemia. Organ transplantation in chronic myeloproliferative disorders is more frequently observed in the context of Budd Chiari and liver transplantation. *Conclusions.* Organ transplantation is possible in the context of chronic myeloproliferative neoplasms after multidisciplinary discussion. More cases are needed to confirm the safe evolution and to obtain supplemental arguments for this procedure.

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SIX YEAR RESULTS OF TARGETED THERAPY FOR CHRONIC MYELOGENOUS LEUKEMIA

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Background. The use of tyrosine kinase inhibitors (TKI) has dramatically changed prognosis in patients with chronic myelogenous leukemia (CML). The estimated median survival associated with TKI administration exceeds 15 years. *Aims.* To evaluate six year results of chronic myelogenous leukemia treatment with tyrosine kinase inhibitors. *Methods.* For the period of 2004 and up until now the 74 CML patients have been observed at the municipal hematology center of Novosibirsk, specifically: 55 patients in the chronic phase (CP), 11 patients in the accelerated phase (AP), and 8 patients with blast crisis. Those 74 patients included 29 men (39.2%) and 45 women (60.8%), with age ranging from 16 to 78 years, and the mean value being 44.7±18.17 years. 51 patients were administered TKI within the first 12 months since the diagnosis of CML was established, of them 43 patients have been receiving treatment for over 6 months (group 1), whereas 8 patients - less than 6 months. In the group of 23 subjects treatment with TKI was initiated in 24 or more months since the diagnosis of CML was established, and those patients had been significantly pre-treated with various medications, comprising Group 2. All patients from the II group prior to Glivec administration were administered various cytostatic agents and interferons: hydroxyurea - 92,8% patients; IFN-α medications in the doses of 3-9 mln IU daily - 78,3%; cycles of low dose cytarabine - 39,1%; bisulphan -13%. Among the 74 patients 67 are administered imatinib mesylate in the doses ranging from 400 to 800 mg daily, whereas 7 patients are administered nilotinib in the dose of 800 mg daily as 2nd line treatment after imatinib resistance or intolerance. *Results.* Administration of a TKI as a single agent in group 1 was followed by a complete clinical and hematological remission (CHR) in 90.7% patients, complete cytogenetic response (CCyR) - in 72,1%, partial response (ph^+ metaphases below 35%) - in 7%, therefore, major cytogenetic responses (MCyR) was achieved in 79.1% patients. Complete molecular response (CMR) was achieved in 55.8% of the studied patients, whereas major molecular response (MMO) - in 25.6%. In group 2 complete clinical and hematological remission was achieved in 52.2% patients, CCyR - in 13%, PCyR - in 4.4%, therefore, MCyR was achieved in 17.4% of the studied patients. Survival was analyzed in patients administered TKI, as compared to patients not administered TKI (data based on the retrospective review of medical records of CML patients observed at Novosibirsk municipal hematology center for the period of 1999-2004). A statistical method of calculating the cumulative fraction of survivals (Kaplan-Meier) was used to evaluate survival, with $P<0,05$ established as the reliability criterion. No medial survival was not established in the group administered TKI, whereas the 10-year survival rate exceeded 70%. In the group administered cytostatic agents median survival 4,1 years, the 10-year survival rate was 9%, $P<0,000001$. *Conclusions.* TKI are considered an effective and safe method of treatment for CML associated with a high MCyR rate in the chronic phase and a high CHR rate in the accelerated phase, therefore considerably improving patients' survival.

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SHWACHMAN-DIAMOND SYNDROME: CLINICAL REPORT OF TWO CASES

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Background. Shwachman-Diamond syndrome (SDS) is a rare autosomal-recessive, multisystem disease characterized by impaired hematopoiesis and exocrine pancreatic insufficiency. Other clinical features include skeletal, hepatic, cardiac disorders and short stature. Patients with SDS have an increased risk for myelodysplasia and acute myelogenous leukemia (AML). Marrow cytogenetic clonal abnormalities, particularly involving chromosome 7 (monosomy 7, der[7], i[7q] and del(20q)) have been reported. Around 90% of SDS patients have biallellic mutations in the Shwachman-Bodian-Diamond syndrome (SBDS) gene, located on chromosome 7 and involved in ribosome biogenesis. The most common cytopenia is neutropenia, with frequent impaired mobility, migration and chemotaxis of neutrophiles. Anemia and thrombocytopenia are less frequent. The cytopenias present in the earlier years of age and the incidence of transformation to AML has been reported as 5% in children, but increases with age (approximate-

ly 25% in adults). *Aim.* to report the diagnosis and evolution of two patients diagnosed with SDS in our center. *Results.* First case report: a 10 year old boy presented with neutropenia at 3 months of age. A bone marrow aspirate was performed, showing normal cellularity without signs of dysplasia nor stop in the granulocytic maturation. The cytogenetic study was normal. In the first year of age, the boy presented transaminitis and mild hepatomegaly, with low fecal elastase levels and mild steatorrhea. An SDS was suspected and molecular analysis was performed, showing a novel mutation in SBDS gene. The relatives were studied confirming the presence of the mutation in maternal grandmother. Subsequently, annually studies of bone marrow aspirate and karyotype have been done, without abnormalities. Currently the patient has no clinical nor analytical symptoms (steatorrhea or neutropenia). Second case report: a girl of 11 years was referred to our center for the study of chronic neutropenia and chromosome 7 abnormality. She had a history of self-limited transaminitis, recurrent otitis media with hearing loss, and diabetes mellitus. The bone marrow aspirate performed showed no dysplastic features and 4% blasts. The cytogenetic study displayed 46, XX [10] / 46, XX i (7) (q10) [10].The blood analysis showed low levels of fecal elastase and steatorrhea. A SBDS gene mutation was found confirming the SDS diagnosis. In the complementary studies to discard other alterations an atrial septal defect was found, requiring surgical correction. During the follow-up of the girl, the bone marrow was found progressively hypocellular without signs of dysplasia and new alterations in the karyotype were found: 46, XX del20q [10] / 46, XX. She has no clinical complications at present. *Conclusion.* SDS should be included in the differential diagnosis of children with chronic neutropenia and / or exocrine pancreatic insufficiency. Fecal elastase level is a good non-invasive test for exocrine pancreatic insufficiency. Once the SDS is suspected, some complementary studies must be done to confirm the clinical diagnosis, including molecular analysis of SBDS gene, a bone marrow aspirate and cytogenetics. In addition, studies of bone marrow during the follow-up are mandatory to exclude the progression to myelodysplasia and AML.

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IMPLICATIONS OF JAK2 MUTATION (V617F) ON THE EVOLUTION AND PROGNOSIS OF CHRONIC MYELOPROLIFERATIVE DISORDERS BCR-ABL NEGATIVE

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This analysis of a lot consisting of 65 subjects, 22 male sex (33.8%) and 43 female (66.2%), 37 subjects with Jak2 mutation positive (57%) and 28 subjects with Jak2 mutation negative (43%), distribution are: polycythaemia vera: 23% (15 subjects), essential thrombocythaemia: 69% (45 subjects) and idiopathic myelofibrosis: 8% (5 subjects). Clinical data were studied: splenic enlargement, the frequency of thrombotic accidents and biological data: serum LDH level, uric acid, platelet number, erythrocyte number, leukocyte number, erythropoietin, degree of marrow fibrosis and therapeutic response. Subjects Jak2-positive men are 12 and have an average age of 56.5 years (42-73 years) and the Jak2-positive women are a total of 25, with a mean age of 65.6 years (32-87 years). Subjects Jak2-negative men are 10 and have an average age of 66 years (50-90 years) and the Jak2-negative women are a total of 18, with a mean age of 59.83 years (29-87 years). We found: massive splenomegaly in 28.5% of subjects Jak2-negative and in 16.2% of Jak2-positive, moderate splenomegaly in 60.7% of subjects Jak2-negative and in 67.6% of Jak2-positive, hypercelularrity on peripheral blood for 39.2% of subjects Jak2-negative and for 45.9% of Jak2-positive; maximum values of serum LDH to 21.4 % of subjects Jak2-negative and 21.69% respectively of the Jak2-positive; moderately elevated values of serum LDH to 71.4% of subjects Jak2-negative and to 70.2% of Jak2-positive; maximum values of serum uric acid at 21.4% of subjects Jak2-negative and at 21.6% of Jak2-positive; moderately elevated values serum uric acid to 60.7% of subjects Jak2-negative and to 54% of Jak2-positive; identical marrow fibrosis. Thrombotic complications (recurrent myocardial infarction, recurrent stroke, arterial and/or deep venous thrombosis or superficial, gangrene, pulmonary thrombosis *in situ*, microvascular infarction of the spleen) had an incidence of 11% in subjects Jak2-negative female and 44% in Jak2-positive women, respectively 10% in subjects Jak2-negative male and 83% in Jak2-positive male. The average age of thrombotic accidents was 55.8 years for subjects Jak2-positive men and 71.25 years for those of Jak2-positive women. For Jak2-negative subjects the average age of thrombosis was 72.5 years for women and 52 years respectively in men. Therapeutic response was identical. Statistical analysis indicates that the only statistically significant difference (P<0.01) is the incidence of thrombotic accidents in subjects Jak2-positive *vs.* Jak2-negative. *Conclusions.* Jak2 mutation have a potential thrombophilic risk.

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THROMBOEMBOLISM PREDICTIVE FACTORS IN POLYCYTHEMIA VERA AND ESSENTIAL THROMBOCYTOPENIA

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Background. Polycythemia vera (PV) and Essential Thrombocythemia (ET) are myeloproliferative chronic disease characterized by the frequent expression of JAK2V617F mutation (90% and 60% respectively). Thromboembolism is a complication that highly impacts on overall survival. *Aims.* A retrospective study of a group of patients affected by PV and ET, classified in accordance with WHO 2008 guide-lines, has been performed. We tried to study potential correlation between thrombotic events and clinical and laboratory variables. *Methods.* We retrospectively analysed the incidence of both arterial and venous thrombosis and their relationship with clinical variables. *Results.* We considered 125 patients:74 female and 51 male. A thrombotic event occurred in 29 patients (23,2%) during the course of their disease: 10 (8%) within the PV group (7 venous and 3 arterial) and only 19 (15,2%) within the ET group (12 venous and 7 arterial). The mean and median time between diagnosis and the occurrence of the thrombotic event were 279 and 172 days respectively. The mean and median time between the star of any treatment for the mieloproliferative disorder and the occurrence of the thrombotic event were 197 and 152 days respectively. Platelets (plt), leukocytes (wbc) and hematocrit (ht) at diagnosis were not statistical different between patients who had a thrombotic event and those who did not. Moreover blood count variables were not different between the diagnosis and the time of the thrombotic event. At the event time all the 29 patients with thrombosis were treated with ASA, and 21 of them received also cytostatics. *Conclusions.* We confirmed the high incidence rate of thromboembolic complications in patients affected by PV and ET. Leucocitosis has been suggested as a risk factor for thrombosis, but in our experience none of them statistically influenced the occurrence of a thrombotic event. This can be due to the relative low number of patients analyzed. The role of leukocytes and platelet number has to be analyzed in prospective studies.

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TREATMENT RESPONSE AND COMPLICATIONS OF PATIENTS WITH ESSENTIAL THROMBOCYTHEMIA

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Background. Essential thrombocythemia (ET) is a myeloproliferative disorder characterized by persistent thrombocytosis. The frequency of JAK2V617F mutation is estimated at approximately 50% of the patients. Thrombotic episodes and bleeding complications are the most frequent events. Secondary malignancies can also occur. Hydroxyurea, anagrelide and interferon-α are available treatments. *Aims.* To asses the treatment response and complications in patients with essential thrombocythemia in our department. *Methods.* We studied retrospectively 97 patients, 41 male and 56 female with a median age of 52 years (27-80). Thrombosis at diagnosis presented in 16/97 patients and hemorrhage in 5/97. Median platelet count was 850×10^6/L (602-2500×10^6/L). Splenomegaly was present in 35 patients and fibrosis in 42. The majority of the patients received anagrelide and 10 interferon-α, 4 received a combined therapy with HU and interferon-α while 7 patients received no therapy. *Results.* Hemoglobin level and platelet count were similar in the two groups of patients. The WBC and PLT count was not correlated with thrombosis or bleeding at diagnosis. Of the patients receiving hydroxyurea, 89% had a response, a significantly higher percentage compared to the other therapy groups (P<0,001). Response was considered when a patient didn't need a second-line therapy in a not otherwise refractory disease. Response at six months (PLT<600.000×10^6/L) was more frequently seen with HU compared with anagrelide (P<0.05). A quick response did not

reduce the incidence of thrombotic or bleeding complications. Secondary malignancies were observed in 4/97 patients (4.12%) with no correlation to HU therapy. *Conclusions.* Our study showed a significant difference between the HU group of patients and those who did not receive HU as front line therapy with regard to therapy response. A response at six months did not affect the incidence of complications.

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CLINICOPATHOLOGICAL DIFFERENCES BETWEEN HIV-POSITIVE AND HIV-NEGATIVE PLASMABLASTIC LYMPHOMA

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Background. Plasmablastic lymphoma (PBL) is a distinct subtype of diffuse large B-cell lymphoma characterized by the malignant growth of lymphocytes with plasmablastic appearance. It is most commonly encountered in individuals with HIV infection and usually has an aggressive clinical course with a poor prognosis. PBL has also been described in patients without HIV infection. *Aims.* The objective of our study was to identify potential clinicopathological differences between HIV-positive and HIV-negative PBL. *Methods.* A comprehensive search using MEDLINE was made looking for HIV-positive and HIV-negative cases of PBL since January 1997 to June 2009. Plasmablastic microlymphoma or HHV-8-associated PBL cases were excluded from our study. Clinicopathological data was gathered, whenever available, and included age, sex, HIV status, clinical stage, site of involvement, presence of B symptoms, bone marrow involvement, EBV expression, Ki-67 expression, outcome and survival in months. Cases were separated according to their HIV status and groups were compared. The T-test was used to compare the age distribution of the groups. The Chi-square test was used to compare sex, stage, oral involvement, B symptoms, bone marrow involvement, EBV expression by EBER or LMP-1 and Ki-67 expression over 80% between groups. Survival curves were obtained using Kaplan-Meier estimates and then compared using the log-rank test. *Results.* Out of 194 identified cases, 181 reported HIV status, 130 were HIV-positive (72%) and 51 were HIV-negative (28%). The mean age for HIV-positive cases was 39 years (SE 10.12) and for HIV-negative cases was 55 years (SE 19.28) (P<0.0001). In terms of sex distribution, male cases were seen in 82% of HIV-positive cases and 62% of HIV-negative cases (P=0.0017). Oral involvement was seen in 64% of HIV-positive cases and 18% of HIV-negative cases (P<0.0001). EBV expression was seen in 69% of HIV-positive cases and 41% of HIV-negative cases (P=0.0068). Ki-67 expression higher than 80% was seen in 79% of HIV-positive cases and 39% of HIV-negative cases (P=0.0055). There were no differences between stage distribution, presence of B symptoms and bone marrow involvement. In terms of survival, HIV-positive cases had a median survival of 13 months (n=66) and HIV-negative cases of 9 months (n=41) (P=0.0015).

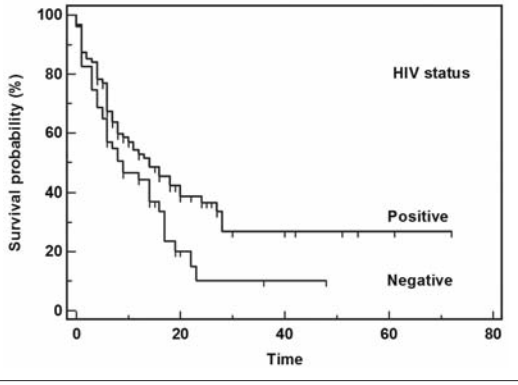

Figure. Survival for PBL according to HIV status.

Conclusions. HIV-positive and HIV-negative PBL have significant clinicopathological differences. HIV-positive PBL tend to affect younger individuals than HIV-negative PBL. It also tends to present more commonly in male patients, with oral involvement, EBV expression in the tumoral cells and higher percentages of Ki-67. Additionally, patients with HIV-positive PBL have a better prognosis than their HIV-negative counterparts. Further research is necessary to clarify these potential differences in order to improve our understanding of the physiopathology and biology of PBL.

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EVALUATION OF CANCER-TESTIS ANTIGENS AS CANDIDATES FOR IMMUNOTHERAPY IN NON-HODGKIN´S LYMPHOMAS USING PROTEIN EXPRESSION AND SPONTANEOUS HUMORAL IMMUNE RESPONSE ANALYSES

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Cancer-testis antigens(CTAs) are expressed in a variety of malignant tumors and in normal adult tissues solely in testicular germ cells. Based on this tumor-associated expression pattern, these antigens are potential targets for immunotherapy. Though carcinomas have been extensively analyzed, less is known about other malignancies such as non-Hodgkin's lymphoma (NHL). *Aims.* To evaluate the potential of tumor specific antigens as candidates for immunotherapy in NHL throughout CTAs protein expression and spontaneous humoral immune response analyses. *Methods.* A tissue microarray was generated from 106 archival cases of NHL. Immunohistochemistry(IHC) was done using a panel of 9 monoclonal antibodies (to the following CTAs): MA454(MAGE-A1), M3H67(MAGE-A3), 57B(MAGE-A4), CT7-33(CT7/MAGE-C1), CT10#5(CT10/MAGE-C2), E978(NY-ESO-1), 219-510-23(LAGE-1 and NY-ESO-1) and #26(GAGE). Spontaneous humoral immune response against 9 CTAs (MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, NY-ESO-1, CT7, CT10, LAGE-1 and GAGE-2) was tested in 97 untreated NHL patient samples, including 63 cases from the TMA cohort, using ELISA technique. *Results.* 12/106(11.3%) NHLs expressed at least 1 of 9 CTAs. DLBCL showed the highest frequency among positive cases. The 12 positive cases were: 9/56 diffuse large B-cell lymphoma(DLBCL), 1/2 anaplastic large cell lymphoma(ALCL), 1/3 lymphoplasmacytic lymphoma and 1/9 peripheral T-cell lymphoma. Among the 12 positive cases, 4 had low, 5 intermediate and 2 high IPI score (1 case was unclassified). Four of these positive cases obtained complete response and 8 partial response or progressive disease using CHOP-like regimens. The most frequently expressed CTAs were GAGE(5.7%), NY-ESO-1(4.8%), CT7(4.8%), MAGE-A1(3.8%) and MAGE-A3(3.8%). In ELISA analyses, 13(13.4%) of 97 LNH sera samples expressed at least 1 of 9 CTAs. Among the 13 positive cases, 6/50 were DLBCL, 2/4 were mantle cell lymphoma, 2/10 were FL, 2/3 were small lymphocytic B-cell lymphoma and 1/2 was ALCL. Five of these patients had low, 4 intermediate and 2 high IPI score (2 cases were unclassified). Seven cases obtained complete response and 6 had partial response or progressive disease, using CHOP-like regimens. The spontaneous humoral immune response was more frequent against NY-ESO-1(5.1%), MAGE-A4(5.1%) and MAGE-A3(4.1%). In the 63 cases with both tissue and sera available, we found 3 positive cases by IHC and 10 positive cases by ELISA; 2/4 IHC-positive cases were also positive by ELISA and 1 IHC positive case was negative by ELISA. We also tested the spontaneous humoral response against CT24(0%), CT45(5.1%), CT46(0%), CT47(7.2%), CT63(1%), CT83(0%), SSX-1(0%), SSX-2(1%), SSX-4(1%), SAGE-1(0%) and XAGE-1(0%) in order to analyze other CTAs in addition to the IHC panel. *Conclusions.* To our knowledge this is the first side by side analysis of the protein expression of CTAs and the autologous serological response in NHL patients. We found low expression (~13%) of CTAs in our series of NHL TMA and sera samples. However, other CTAs should be explored to establish the real potential of these antigens as candidates for immunotherapeutic approach in NHL. Considering that most of advanced stage low-grade lymphomas, mantle cell lymphoma and T-cell lymphomas are still incurable diseases and need the development of new therapeutic approach, these tumors should be further explored as to the presence of CTAs.

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ESTABLISHMENT, CHARACTERIZATION AND PHARMACOLOGIC EVALUATION OF A UNIQUE IN VITRO AND EX VIVO STUDY MODEL FOR B-CELL NODAL MARGINAL ZONE LYMPHOMA

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Background. A number of cell lines derived from patients with B-cell non-Hodgkin lymphomas (B-NHL) have been established over the last decades and served as research tools for *in vitro* studies focusing on the

mechanisms of lymphomagenesis as well as testing of new therapeutic approaches for these patients. However, the established B-NHL cell lines have been derived mostly from aggressive, high proliferating B-NHL, while cell lines derived from patients with "low-grade"B-NHL types are largely lacking. *Aims.* This study aimed to develop and characterize a novel *in vitro* and *ex vivo* study model for B-cell nodal marginal zone lymphoma (NMZL). *Methods.* Primary tumor cells obtained from a lymph node biopsy specimen were put in culture, and the suspension cells were maintained under culture conditions in RPMI medium with increased concentration of FBS (20%). When the cells overcame the cell culture crisis and started to proliferate, 10X106 cells were injected subcutaneously in SCID mice, which subsequently developed xenografts. Cells from xenografts were sub-cultured and re-injected in new SCID mice and this procedure was repeated three times. The established cell line named "LGH1"and its first xenograft (xLGHp1), were characterized by immunophenotypic (flow cytometry), immunohistochemical and cytogenetic analysis. A number of drugs were tested for activity against both the LGH1 primary cells and the cells originated from the xenograft (xLGHp1) using the Sulforhodamine B, trypan blue exclusion, MTS and other assays. *Results.* Cell line was derived from a 66-year old patient presented with non-bulky left inguinal lymphadenopathy and no other symptoms or physical findings. Thoracic and abdominal CT scans as well as upper GI endoscopy were negative. A bone marrow biopsy and immunophenotype were negative. Both blood and bone marrow samples were negative for clonal immunoglobulin rearrangements by PCR. Lymph node biopsy was consistent with a NMZL with a cell proliferation index (Ki-67) of 10-15%. Conventional clinical stage was IA; PET-based stage was IIA. Immunophenotypic analysis (flow cytometry) and immunohistochemistry performed on cell blocks from cultured cells were typical for NMZL consistent with the original histologic diagnosis. LGH1 cell karyotype was normal (46, XY). Mutation analysis revealed wild-type p53 gene. Pharmacologic evaluation showed no significant differences regarding their response to the drugs between the LGH1 and xLGHp1 cells. The most active drugs in this NMZL model were found to be the two anthracyclines doxorubicin and epirubicin with growth inhibiting activities at 1μM. In addition, LGH1 cells responded significantly to nutlin 3A (10μM), a potent MDM2 inhibitor that stabilized and activates wild-type p53. Moderate biologic responses to rapamycin were observed at a concentration of 40nM. Fludarabine, one of the established drugs used for lymphoma treatment, was found to be almost inactive as it demonstrated marginal activity at the highest concentration tested (100μM). *Conclusions.* A unique *in vitro* and *ex vivo* model for NMZL was established and characterized, thus providing a research tool to perform new mechanistic studies in order to shed light on the NMZL pathogenesis. Furthermore, pharmacologic studies using LGH1 may lead to the development of novel targeted therapies for patients with NMZL.

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INHIBITION OF THE INTRINSIC APOPTOSIS PATHWAY IN FOLLICULAR LYMPHOMA IS CAUSED BY DYSREGULATION OF THE MITOCHONDRIA

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Aim. Inhibition of the apoptosis cascade is an important cause of therapy resistance in follicular lymphoma (FL). In this study we investigate possible mechanisms in the apoptosis pathway and expression levels of apoptosis related genes that may be responsible for differences in chemotherapy sensitivity between FL patients. *Methods.* Isolated lymphoma cells of 30 FL patients and 5 samples of centroblasts and centrocytes of healthy donors were investigated for their expression levels of apoptosis related genes using RT-Multiplex Ligation dependant Probe Amplification (RT-MLPA) analysis. Functional analysis of the intrinsic caspase 9 mediated pathway was performed using immunohistochemistry and FACS analysis. *Results.* All FL samples tested demonstrated inhibition of the intrinsic apoptosis pathway and resistance to etoposide. Using RT-MLPA analysis different expression patterns of apoptosis regulating genes were observed: one group characterized by extremely low pro-apoptotic gene expression, and one group with relatively low pro-apoptotic and high anti-apoptotic gene expression. Functional analysis revealed very low spontaneous caspase 9 activity and impermeability of the mitochondrial membrane in FL patient cells. Furthermore, Bcl-2 and/or Bcl-XL expression were high in the majority of the FL samples. In these cases, apoptosis could be restored using a Bcl-2/Bcl-XL antagonist. *Conclusion.* We conclude that in most FL patient samples the intrinsic caspase 9-mediated pathway is inhibited upstream by disruption of the function of the mitochondria. Apoptosis can be restored by treatment with a Bcl-2/Bcl-XL antagonist which therefore might be an alternative therapy option for FL.

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P-GLYCOPROTEIN EXPRESSION IS ENHANCED IN EPSTEIN-BARR VIRUS (EBV)-INFECTED T OR NK CELLS AND MAY CAUSE DRUG RESISTANCE OF CHRONIC ACTIVE EBV INFECTION

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Background. Epstein-Barr virus (EBV) infects B cells and occasionally T or NK cells, causing EBV-positive T/NK-cell lymphoproliferative disorders (EBV-T/NK-LPD). Chronic active EBV infection (CAEBV), a form of EBV-T/NK-LPD, is a rare disease accompanied by persistent infectious mononucleosis-like syndrome and associated with high titers of antibodies against EBV. CHOP is usually selected as first-line treatment. However, it has resulted in limited effects while prognosis remains very poor. *Aims.* To clarify the molecular mechanism of chemoresistance of CAEBV, we focused on multi-drug resistance 1 (MDR1) gene expression. This gene encodes for P-glycoprotein (P-gp), which is a well-known drug efflux pump. It was previously reported that cells of extranodal NK/T-cell lymphoma (ENKL), a form of EBV-T/NK-LPD, expressed P-gp, resulting in high resistance to CHOP. We analyzed its expression and function in cells derived from CAEBV patients. *Methods.* CAEBV was diagnosed according to the criteria suggested by Okano M. *et al*. (Am J Hematol 80:64-9, 2005). To detect and isolate EBV-infected cells, T and NK cells were separated using magnetic beads from peripheral blood mononuclear cells of CAEBV patients. DNA was extracted and EBV-DNA was quantified using a real-time quantitative PCR assay. The clonality of EBV was determined by southern blotting using a terminal repeat probe. Transcription of MDR1-RNA and expression of P-gp was examined by RT-PCR and flow cytometry (FCM), respectively. The fluorescent compound rhodamine 123 was used as a P-gp substrate to examine the function of P-gp. SNK6, derived from ENKL, and MD901, a B-cell lymphoma cell line, were used as positive and negative controls, respectively. The study was approved by the ethical committee and written informed consent was obtained from each patient. *Results.* Four CAEBV patients were diagnosed and treated with CHOP in our hospital. Patient age at the start of treatment ranged from 24 to 48. The infected cells were T cells in all the patients ($CD4^-$ or CD8-positive in 1 or 3 cases, respectively). Monoclonal expansion was confirmed in all patients. After treated with CHOP, 3 patients demonstrated no effects, and only 1, CD4-type patient, achieved 1-log reduction of EBV-DNA in peripheral blood. EBV-infected cells derived from 13 CAEBV patients (aged 8-45 years; 6 male, 7 female; 2 $CD4^-$, 4 $CD8^-$, 6 $CD56^-$, and 1 γδ-cell types) were investigated. Transcription of MDR1 was detected in all patients. P-gp expression was confirmed by FACS. The mRNA level of MDR1 was highest in CD56-positive cells and lowest in $CD4^+$ cells, which was compatible with the effect of CHOP in our patients. In addition, function of P-gp was confirmed by rhodamine 123 efflux assay in 5 patients. Efflux was completely inhibited by treatment with a P-gp inhibitor, cyclosporin A, which induced CAEBV cell death synergistically with doxorubicin. *Conclusion.* EBV-infected T or NK cells expressed P-gp, which may contribute to multi-drug resistance of CAEBV. Differences in expression level between the infected cells were observed, suggesting that a different treatment strategy should be considered for each disease type. Further studies analyzing more cases should be performed to establish the optimal treatment.

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DOWNREGULATION OF CD19, CD20 AND CD22 SURFACE EXPRESSION ON MANTLE CELL LYMPHOMA BY LENALIDOMIDE

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Background & Aims. Lenalidomide is a second-generation immunomodulatory drug with clear activity against mantle cell lymphoma (MCL) as a single agent. Recent clinical trials have investigated combining lenalido-

mide with the anti-CD20 monoclonal antibody rituximab in an attempt to further improve efficacy in MCL. However, lenalidomide has been shown *in vitro* to downregulate CD20 surface expression on primary chronic lymphocytic leukaemia cells thereby diminishing rituximab-mediated apoptosis and antibody-dependent cellular cytotoxicity. With the increasing use of rituximab for the treatment of MCL together with the advent of new therapeutic monoclonal antibodies against alternative B cell markers such as CD19 and CD22, we evaluated the effects of lenalidomide on the surface expression of these antigens using the MCL cell line Granta 519. *Methods.* Granta 519 cells (0.5×10^6/mL) were incubated in complete culture medium with or without dimethyl sulphoxide (DMSO, vehicle control), thalidomide (1 μg/mL or 10 μg/mL) or lenalidomide (0.26 μg/mL or 10 μg/mL) for up to 72 hours. The lower concentrations of thalidomide and lenalidomide were chosen to represent therapeutic plasma levels achievable in patients. CD19, CD20 and CD22 surface expression on Granta 519 cells was quantified by flow cytometry after 24, 48 and 74 hours. Results were expressed as mean percentage change compared to control (complete culture medium alone), and Students t test was used to determine differences between the treatment arms at each timepoint. Two-tailed p values <0.05 were considered statistically significant. *Results.* Incubation for up to 72 hours with thalidomide did not result in any significant change of CD19, CD20 or CD22 surface expression on Granta 519 cells. In contrast, lenalidomide 10 μg/mL significantly downregulated CD20 surface expression at 24, 48 and 72 hours with a mean reduction of 14.0% (P=0.0371), 28.1% (P=0.0219) and 28.4% (P=0.0003) respectively. Moreover, lenalidomide 0.26 μg/mL also significantly downregulated CD20 surface expression at 72 hours with a mean reduction of 22.0% (P=0.0043). Preliminary analysis of CD19 surface expression showed a similar pattern of downregulation following treatment with lenalidomide 10 μg/mL for 48 hours (mean reduction 22.7%, P=0.0372) and lenalidomide 0.26 μg/mL for 72 hours (mean reduction 17.2%, P=0.0223). Preliminary analysis of CD22 surface expression also revealed significant downregulation with lenalidomide 10 μg/mL at 48 hours (mean reduction 30.9%, P=0.0254) as well as a trend towards downregulation with lenalidomide 0.26 μg/mL (mean reduction 19.6%, P=0.0821). Summary/*Conclusion.* Lenalidomide at a concentration achievable in plasma (0.26 μg/mL) has the ability to downregulate the B cell markers CD19, CD20 and CD22 on the MCL cell line Granta 519. This has clear implications when considering the combination of lenalidomide with monoclonal antibodies directed against these surface antigens, and would suggest that a sequential approach to administering these agents may be more efficacious than a simultaneous one. Further confirmatory work utilising primary MCL cells isolated from patients is ongoing.

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DIFFUSE LARGE B CELL LYMPHOMA: DIFFERENCES IN BIOLOGICAL CHARACTERIZATION OF THE NODAL VERSUS EXTRANODAL PRESENTATION BY IMMUNOHISTOCHEMISTRY

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Introduction. We hypothesized that diffuse large B cell lymphoma (DLBCL) with primary extranodal origin may be immunophenotypically different from primary nodal origin. Primary extranodal lymphoma cells may originate from activated B cell rather than germinal center B cell. We evaluated the clinicopathological features of patients with diffuse large B cell lymphoma (DLBCL) according to the primary site, lymph node or different extranodal organs of the disease. *Patients and methods.* Immunohistochemistry was performed for detection of CD10, Bcl-6, Bcl-2 and MUM1 in paraffin-embedded tissue from 123 patients with DLBCL. We classified cases into germinal center B-cell like (GCB) and non-GCB groups using the immunohistochemical expression of CD10, bcl-6, or MUM1 by Hans' algorithm. Lymphomas presenting in extranodal organs with no or only minor lymph node involvement were considered primary extranodal and those lymphomas with extensive disease involving both nodal and extranodal sites were considered nodal. Statistical analysis was performed using χ^2 and Fisher's exact tests. Survival data were analyzed by the Kaplan-Meier method and compared using a log-rank test. *Results.* Thirty-eight (31%) of the 123 cases were of germinal center phenotype ($CD10^+$ or $CD10^-$, $Bcl\text{-}6^+$, and $MUM1^-$) and 85 (69%) were of non-GCB phenotype (all CD10- and either $bcl\text{-}6^-$, or $bcl\text{-}6^+$ and $MUM1^+$). Fifty-one patients (42%) presented at primary extranodal sites. The distinction according to the extranodal sites was as follows; GI tracts (29; 14 cases of stomach and 15 cases of Intestine); testis/ovary/adrenal (4); skin/soft tissue (3); thyroid/parotid (3); bone/bone marrow (2); pancreas (2); cervix (2); and prostate (1). Five patients showed multiple extranodal lymphomas. Of these, 16 (31%) was classified as GCB subtype and 35 (69%) was non-GCB subtype. 3 (20%) in the intestines were GCB subtype and 7 (50%) in the gastric DLBCL were of GCB subtype (P=0.12). 72 patients had a primary nodal DLBCL. Of these, 22 (31%) was classified as GCB subtype and 50 (69%) was non-GCB subtype. There was no difference in frequencies of GCB and non-GCB subtype between primary extranodal and primary nodal DLBCL. However, Bcl-2 expression is less frequent in primary extranodal disease (P=0.008). Among stage I or II patients, primary extranodal DLBCL showed a trend toward better overall survival than primary nodal DLBCL (P=0.09) and among stage III or IV patients, there was no survival difference between the two groups. *Conclusion.* Consistent with previous reports, the GCB subtype of DLBCL was less frequent in Asian. There was no difference in GCB and non-GCB phenotype in primary extanodal and primary nodal DLBCL in our patient population. Further studies are warranted to elucidate the molecular differences between primary extranodal and primary nodal lymphoma.

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CLONAL EVOLUTION IN A CASE OF HEPATOSPLENIC T CELL LYMPHOMA

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γHepatosplenic T-cell lymphoma (HSTCL) is an uncommon disease. It is characterized by involvement of the spleen, liver and often bone marrow (BM). HSTCL affected Patients usually show hepatosplenomegaly and cytopenias; lymphadenopathy is minimal or absent. The disease has a progressive clinical course and poor prognosis. Here, we describe the case of a 51-years old gamma-delta HSTCL affected female.In May 2006, the Patient showed massive hepatosplenomegaly; blood counts revealed mild pancytopenia. The Patient underwent splenectomy, hepatic biopsy and BM analysis. The three parenchymas were infiltrated by a population of abnormal lymphoid cells. Cytofluorimetric analysis of BM, spleen and liver biopsy showed $CD45^+$ $CD2^+$ $CD3^+$ $CD7^+$ $CD56^+$ TcR-$\gamma\delta^+$ $CD5^-$ $CD4^-$ $CD8^-$ neoplastic cells. Molecular analysis, performed by denaturing high-performance liquid chromatography (DHPLC), showed clonal rearrangement of TcR-γ chain gene on the same samples. Sequencing analysis of both DNA strands confirmed DHPLC results, by allowing us to identify the monoclonal rearrangement Vγ10-JγP1. Therapy consisted of six CEOP-14 regimen. In December 2006 a complete remission (CR) was demonstrated by immunological, histological and molecular analysis of BM and liver biopsy. CR lasted 21 months; in February, 2008 mild pancytopenia appeared. Positron emission tomography - computed tomography (CT-PET), immunophenotipic and histological studies were negative; TcR-γ clone was not detectable. In September 2008 anaemia worsened, mild elevation of liver enzyme and abnormal serum ferritin appeared. Histological BM biopsy was positive for lymphoma infiltration, despite negative cytofluorimetric and morphological analysis. Histological and cytofluorimetric analysis of liver biopsy were both positive for T lymphoma with $CD45^+$ $CD2^+$ $CD7^+$ $CD56^+$ $CD3^-$ $CD5^-$ $CD4^-$ $CD8^-$ TcRαβ$^-$, TcRγδ$^-$ large abnormal lymphocytes. Molecular study showed the Vγ10-JγP1 and an additional Vγ11-JγP1 clonal rearrangement on bone marrow.The Patient was treated with four cycles of DHAP reaching CR (at liver level as well); in March 2009, she underwent auto-TMO. In August 2009, a second relapse occurred. On liver biopsy, histological study showed absence of neoplastic cells, but cytofluorimetric analysis was positive for large neoplastic cells. BM biopsy was positive for lymphoma infiltration. The Patient was treated with GEMOX regimen. In November 2009, sequencing analysis on bone marrow biopsy detected only the Vγ11-JγP1 clone; cytofluorimetric analysis identified a NK-like immunophenotype cell population; one cycle of Nelarabine was administered but the Patient died on December 2009. With the aim to add data in a rare disease like HSTCL we want to point of: (a) immunophenotypic changes from a T-cell ($CD45^+$ $CD2^+$ $CD3^+$ $CD7^+$ $CD56^+$ TcRγδ+ $CD5^-$ $CD4^-$ $CD8^-$, at diagnosis) to a NK-like ($CD45^+$ $CD2^+$ $CD7^+$ $CD56^+$ $CD3^-$ $CD5^-$ $CD4^-$ $CD8^-$ TcRγδ-, at second relapse) immunophenotype; (b) the evidence of two different clonal rearrangements of TcR-γ- chain gene (Vγ10-JγP1 and Vγ11-JgP1).

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PROINFLAMATORY CHEMOKINE GENE EXPRESSION INFLUENCES SURVIVAL OF PATIENTS WITH NON-HODGKIN'S LYMPHOMAS

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Background. Cell migration, surviving and proliferation creates base for all physiologic and pathologic process in human body. All those reactions are regulated by complex chemokine network, that guides lymphocytes homing, chemotaxis, adhesion and interplaying between immunologic system response cells. Chemokines also are responsible for metastatic dissemination of cancers, including Hodgkin's and non-Hodgin's lymphomas. *Aim.* The purpose of the study was to determinate chemokine gene expression (CXCL8, CXCL10, CCL2, CCL3, CCL4 and CCL5) in lymphoma lymph nodes comparing to their expression in reactive lymph nodes. We also wanted to analyze the influence of chemokines gene expression on lymphoma patient survival. *Methods.* Chemokine gene expression was evaluated in 37 lymphoma lymph nodes (16 women and 21 men, aged 18-81 years; median age 43 years) at the moment of diagnosis. In 25 samples of reactive lymph nodes (taken from 15 women and 10 men; aged 18-59; median age 32 years) expression of chemokine genes was also studied. Gene expression of chemokines CXCL8, CXCL10, CCL2, CCL3, CCL4 and CCL5 was measured by PCR method and estimated in arbitrary units (AU) from 0 to 3 AU points scale. PCR was conducted using primer pairs for CCL2 (sense TCC AGC ATG AAA GTC TCT GC, antisense TGG AAT CCT GAA CCC ACT TC, 245 bp), CCL3 (sense GTC ATC TTC CTA ACC AAG CG, antisense TGT GGC TGT TTG GCA ACA AC, 229 bp), CCL4 (sense AGG AAG CTT CCT CGC AAC TT, antisense AGT CCT GAG TAT GGA GGA GA, 244 bp), CCL5 (sense CAT TGC TAC TGC CCT CTG CG, antisense GGG TTG GCA CAC ACT GTT CG, 192 bp), CXCL8 (sense TTG GCA GCC TTC CTG ATT, antisense AAC TTC TCC ACA ACC CTC TG, 403 bp), CXLC10 (sense TGG CAT TCA AGG AGT ACC TC, antisense TGT AGG GAA GTG ATG GGA GA, 326 bp), β-actin (sense GGG TCA GAA GGA TTC CTA TG, antisense GGT CTC AAA CAT GAT CTG GG, 250 bp). Statistical analysis was performed using the CSS Statistica for Windows (version 7.0) software. Probability values <0.05 were considered statistically significant and those between 0.05 and 0.1 as indicative of a trend. *Results.* Lower expression of CXCL10 gene was found in lymphoma lymph nodes comparing to reactive lymph nodes (P=0.002). In lymphomas CCL2 (P=0.0008) and CCL3 expression in was higher than in reactive lymph nodes. Patients with high expression of CCL2 (P=0.004) and CXCL10 (P=0.002) had significantly shorter survival. *Conclusions.* CCL2 and CXCL10 expression seems to be important in non-Hodgkin's lymphomas, as it has shown to have predictive value on patients' survival.

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IDENTIFICATION OF T(11;14)(Q13;Q32) IN VARIOUS HEMATOPOIETIC LINEAGES OF MANTLE CELL LYMPHOMA PATIENTS. JOINT APPLICATION OF FLUORESCENCE-ACTIVATED CELL SORTING AND FLUORESCENCE IN SITU HYBRIDIZATION

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Background. Mantle cell lymphoma (MCL) is a distinct incurable B-cell neoplasm with a median survival of 3 to 5 years. t(11;14)(q13;q32) is the hallmark of the disease leading to overexpression of protooncogene cyclin D1. Chemoresistance to conventional treatment and continuous disease relapses even after high dose therapy and auto stem cell transplantation define the clinical course of the disease. A possible cause of this frequent recurrences may be that t(11;14)(q13;q32) occurs in early hematopoietic precursors, capable of multilineage differentiation. *Aim.* evaluation of t(11;14)(q13;q32) in different hematopoietic lineages of MCL patients. *Methods.* Bone marrow mononuclears from 4 MCL patients were sorted by Fluorescence-Activated Cell Sorting (BD FACSVantage SE) to divide the following lineages of hematopoiesis: $CD45^+CD34^+$ (progenitor cells); $CD45^+CD5^+CD19^+$light chain Ig (mantle cell lymphoma); $CD45^+CD5^-CD19^+$ (normal B-cells); $CD45^+CD14^+$ (monocytes); $CD45^+CD3^+$ (T-cells); $CD45^-GlyA^+$ (erythrokaryocytes) and granulocytes by light scattering, without range including $CD14^+CD45^+$ cells. The purity of sorted cells was checked by flow cytometry (BD FACSCanto II) and directly correlated to the number of sorted cells - if the cells were more than 50 thousands the purity was more than 92% (usually more 97%), but if the cells were less than 20 thousands the purity was 80% (never the less). After sorting cells in the test-tube were washed from the salt solutions, fixed in methanol and glacial acetic acid mixture and layered onto slides by cytospin cytocentrifuge (Cellspin II Tharmac) in 8 minutes, 1000 rpm. Furthermore, the probes have been denatured at 75oC for 6 minutes, and hybridized with dual fusion LSI IGH-CCND1 probes (Vysis Inc.) at 37oC for 17-20 hours. Signals were visualized with Olympus microscope with triple filter cube (DAPI / FITC / TexasRed). *Results.* In sorted mantle cell lymphoma cells t(11;14)(q13;q32) was present in more than 80%, whereas in cells of all other lineages including normal B-cells t(11;14)(q13;q32) was less than 0,5%. However, direct assessment of t(11;14)(q13;q32) in $CD34^+CD45^+$ cells and in normal B-cells was difficult due to the small population of these cells and further studies are required for more definite conclusion. *Conclusions.* Out data suggests the absence of t(11;14)(q13;q32) in pluripotent stem cells, and the direction of our future research would be the search for t(11;14)(q13;q32) in early B-cell lymphoid progenitors.

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CD10 EXPRESSION IN LYMPH NODE STROMAL CELLS DOES NOT SIGNIFICANTLY INFLUENCE GENE EXPRESSION ANALYSIS OF CD10 POSITIVITY OR NEGATIVITY OF A TUMOR

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Backgroung. Gene expression profiling (GEP) is a method widely used in science. For example, diffuse large B-cell lymphoma (DLBCL) was divided into three groups with the method. One of the important molecules in DLBCL subclassification is CD10 and its expression in tumour cells. Complete paraffin slides without using laser dissection are commonly used in GEP examination, however, CD10 molecule is sometimes expressed also in nonmalignant stromal cells of a lymph node. *Aims.* Our aim was to analyze whether CD10 positivity of stromal cells in CD10 negative tumours can elevate the GEP result so much that such a sample could be considered a CD10 positive tumour. *Methods.* 69 formalin-fixed, paraffin embedded (FFPE) samples of verified DLBCLs were included in the study. RNA extraction and RTqPCR analysis were performed following the protocol we previously published. Only cases with 80% and more malignant cells in the slide were included. Immunohistochemical CD10 staining using standard methods was performed on FFPE slides. The percentage of CD10 positive cells was evaluated in the malignant and stromal cell population in each sample. Cut off of 10% was used as a criterion of positivity. *Results.* Tumors were divided into four groups accordig to their tumor(T)/stromal(S) positivity and their combinations: Group1 T^-/S^-, group 2 T^-/S^+, group 3 T^+/S^- and group 4 T^+/S^+. CD10 positive and negative tumours were significantly different from each other (P<0,001), regardless of the positivity or negativity of the stroma. Groups 1 and 2 were not significantly different in CD10 gene expression (P=0,096). Groups 2 and 3 were significantly different (P=0,012) from each other. Group 2 differed significantly also from group 4 (P<0.001). Positive tumours did not show significant difference between stromal positive and negative cases (P=0,820).*Summary/Conclusions.* CD10 stromal positivity slightly elevates CD10 level measured with real-time quantitative polymerase chain reaction (RTqPCR), however, such elevation is not statistically significant and does not reach the expression level of positive tumors. There is an obvious difference in CD10 gene expression between T^-/S^+ and T^+/S^- samples and between T^-/S^+ and T^+/S^+ ones.

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1542

ANGIOGENESIS IN INDOLENT NON-HODGKIN'S LYMPHOMA (NHL)

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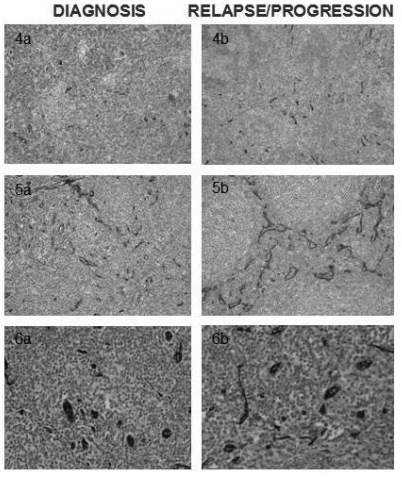
Background. The role of angiogenesis in lymphoma is still under active investigation. Microvessel density (MVD) measures lymphoma neovas-

cularity with a wide range of results reported in the literature. Indeed, the clinical predictive value of MVD with respect to underlying lymphoma subtypes remains unclear. MVD at diagnosis is more represented in aggressive histotypes than in indolent ones. Increased vascularization is associated with both poor outcome and resistance to therapies in diffuse large B-cell lymphoma, independently from the IPI score. Few data are available in relapsed/refractory indolent NHL. Stromal cells (mostly monocytes and macrophages) and tumor microenvironment contribute to neo-angiogenesis with several authors reporting possible correlations among macrophage infiltration, neovascularity and prognosis. *Aim.* The aims of this study are the analysis of neoangiogenic pattern of indolent NHL patients (pts) and the role of stromal cells in neovascularisation at diagnosis and relapse/progression disease in order to identify a subset of pts that could benefit by integrated antiangiogenic treatment. *Patients and methods.* Nodal and bone marrow biopsies from 6 follicular (FL) and 7 small lymphocytic (SLL) NHL's pts were selected and studied at diagnosis and disease relapse/progression (9 males and 5 female; mean age at diagnosis 59.8 yrs; 13/13 stage III/IV; low/intermediate FLIPI 7/13 pts, high risk FLIPI 6/13 pts, 8 relapsed pts with median PFS 33.37 months and 5 refractory). We evaluated MVD both by immunohistochemistry (anti-CD34) and morphometric analysis (vascular hot spots, vhs/field) and the monocytic-macrophagic infiltrating cells (anti-CD68). *Results.* At diagnosis we observed an angiogenic activity in all nodal samples with an homogeneous vascular distribution in SLL and perifollicular in FL (mean of 27.50 vascular hot spots (vhs)/field; median of 20 vhs/field in nodal biopsies). Moreover, at onset patients with low/intermediate prognostic risk showed a higher vhs/field. The number of vhs/field increased in all cases at relapse/progressive disease (mean 39,6; median 44 vhs/field) with statistical significance (T-test P=0.0049; Wilcoxon signed-rank test P=0.03) (Figure 1). On the contrary, no significant angiogenic activity was reported in bone marrow neither at diagnosis nor at relapse/progression. The distribution of monocytic-macrophagic infiltrate was heterogeneous in nodal samples with a focal or diffuse pattern and with a different match with the neovessels The percentage of monocytic-macrophagic infiltrate showed a poor increment at relapse/progression and seemed to lack any correlation with clinical features. *Conclusions.* These preliminary data could justify the employment of angiogenesis analysis in the prognostic stratification of indolent NHL and hypothesize the use of anti-angiogenic drugs in patients relapsed or refractory to treatment. Larger series are warranted to confirm these data.



CASE	DIAGNOSIS	RELAPSE /PROGR.	DFS mts
4	20	50	13
5	50	50	28
6	20	38	28

Figure 1, MVD (vhs/field) in FL NHL.

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DIFFERENTIAL F-18 FDG UPTAKE AFTER 1 CYCLE CHEMOTHERAPY IN NK T-CELL LYMPHOMA: COMPARISON WITH DIFFUSE LARGE B CELL LYMPHOMA (DLBL)

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We prospectively compared the F-18 FDG uptake after 1 cycle between NK T-cell and DLBL. Five patients (M: F = 5: 1, 45 +/- 8.76 year) with NK-T cell lymphoma and 14 patients (M: F = 11: 14, 55.5+/- 14.15) with DLBL were prospectively enrolled in this study from May 2005 to Feb 2007. The protocol was approved by our Institutional Review Board and all patients gave informed written consent. F-18 FDG PET/CT was performed before to chemotherapy (baseline) and then was repeated after 1 cycle chemotherapy. Semi quantitative and visual analyses were performed. We selected 1-9 regions of interest (ROIs) from each patient, for a total of 78 ROIs from all 19 patients. (1) the corrected SUV (SUV cor) was calculated by subtrating the SUV of surrounding normal tissue of tissue opposite the ROIs from the SUVmax of the ROIs, as the SUVmax alone does not correctly reflect tumor activity in regions in which the SUV is low or the background uptake is high. The decrease rate of SUV [RI (%)]= (baseline max SUVcor (SUV1) - after 1 cycle max SUVcor (SUV2)/ SUV1) × 100. (2) Visible change (%) in the extent of FDG uptake in longest dimension was calculated. (the decrease rate of metabolic extent [DI (%)] = baseline metabolic extent (cm2)(D1)-metabolic extent (cm2)((D2) /D1 X 100). A total of 52 ROIs [23 NK T (n=5), 42 DLBL (n=15),] were evaluated in 19 patients. (1) In 23 NK-T, SUV1 ranged 3.97 to 7.64 (mean 5.72; SD 1.02), SUV2 ranged 3.96 to 7.52 (mean 5.66; SD 1.01), RI ranged 0.2 to 2.71 (mean 1.12; SD 0.88) and decreased rate ranged 97 to 100 % (mean 99; SD 0.2). In 23 NK-T, D1 ranged 1.5 to 6.09 (mean 3.58; SD 1.48), D2 ranged 0.47 to 3.37 (mean 1.92; SD 0.16), and LI ranged 16.67 to 75 (mean 45.76; SD 17.23). 2) In 42 DLBL, DI ranged 2.19-10.45 (mean 5.26; SD 2.02) cm, L2 ranged 0 to 0.78 (mean 0.21, SD 0.23) cm and LI ranged 91 to 100 (mean 96, SD 3.06) %. There were significant difference of the DI between NK-T and DLBL (P<0.0001, non paired student t-test).

This study shows significant different changes in FDG uptake between the NK/T and DLBL after first cycle chemotherapy.

1544

THE ROLE OF HELICOBACTER PYLORI IN OCULAR MALT LYMPHOMA: A REVIEW OF OUR SERIES

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Background. The MALT lymphoma was firstly described in 1983 by Isaacson and Wright(1) in two cases with digestive system involvement and similar features to mucosa-associated lymphoid tissue. It is a well-defined nosologic entity in the new classification by the WHO(2). A close relationship between *Helicobacter pylori* infection and the development of gastric MALT lymphoma has been observed several years ago(3-6) and their response to antibiotherapy(7-8). *Aims.* Starting from a case in a patient with a gastric and conjunctival MALT non Hodgkin lymphoma that showed sensitivity to antibiotic treatment (Figure 1) we looked for some similar published cases. We found an article of a Korean research group who showed the 100% presence of *H.pylori* by PCR analysis in 15 cases of conjunctival MALT lymphoma(9). We stablished the hypothesis of a cause-effect relationship between the H.pylori infection and the conjunctival MALT lymphoma based on the observed response to antibiotherapy in our patient. We developed a retrospective study in order to determine presence of genomic material of *H.pylori* by PCR analysis in biopsies samples of gastric and orbital MALT lymphomas in our cases. *Methods.* We determine the presence of genomic material of *H.pylori* by PCR analysis in samples from seven gastric biopsies and five orbitals tissue with MALT lymphomas. DNA extraction: after the deparaffination with xylenes, we employed QIA amp DNA Mini Kit QIAGEN GmbH, D40724 Hilden. Helicobacter DNA was used as an amplification control. It was obtained from a culture by mechanical lysis in MagNALyser and extraction in MagNA Pure Compact. PCR in real time: in 20 uL of the following mixture of capilar reaction: 0,5 mM of each primer (HP-FOR, 5'-TTA TCG GTA AAG ACA CCA GAA A-3', y HP-REV, 5'-ATC ACA GCG CAT GTC TTC-3') 2.4 mM MgCl2, 2 ul FastSart DNA Master SYBRE-Gren I and 10 ul DNA from the samples and the control. *Results.*Our results were two negative test and five positive results in gastric mucosa and five negative results in the five biopsies of ocular adnexal tissue. *Summary/Conclusions.* Due to the disagreement results between our cases and published Korean series, we checked the methodology that both groups had employed trying to explain this disaccord. As a result, we found an important difference between the used primers. They chose primers from the vacA region, whereas we used primers from the ureC region.

We redirected our study by employing the same primers that Korean group but we obtained the same results as before. We think that this disagreement could be explained through the different prevalence of the *H.pylori* infection in the Asian region and genetic differences between studied populations. Because of these conditions these studies are not comparable. Would require further meta-analysis including larger samples from several geographical areas and include more clinical data to compare results and draw conclusions about the role of *Helicobacter pylori* in non gastric MALT lymphomas.

Figure 1.

1545

AFFECT OF CD20 STRENGTH ON B CELL LYMPHOMA PATIENTS' BACKGROUND AND PROGNOSIS

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Background and Aim. Anti CD20 antibody, thus Rituximab have improved the disease free survival and overall survial of several types of B cell lymphoma, which are expressed CD20 on the surface and in the cytoplasma. Expression of CD20 analized by flow cytometry varies from null, weak to strong. Affect of CD20 strength on lymphoma therapy have not well elucidated in spite of the clnical importance of this protein. *Patients, materials and methods.* 214 cases with newly diagnosed B-cell lymphoma were analyzed. All of them were biopsied, also analyzed by flow cytometry, and were newly diagnosed as B cell lymphoma from January in 2002 to April in 2009, in our institute. Informed consent was obtained. The biopsy specimens were fixed in formalin and stained with Hematoxylin-Eosin, and were also immunostained. The histological subtypes were defined according to the World Health Organization Classification Ver 3. The mean florescence intensity of CD20 and CD19 was determined by FCM, and The cytoplasmic expression of CD20 was determined by immunohistochemistry(IHC). 1) The cases were categolized as follows; Group A: CD20 negative, B: CD20/19 less than 20%, C: CD20/19 20-50% and D: CD20/19 more than 50%. And background of patients, pathological diagnosis, primary lesion, expresion of CD5, 23, IgM, T cell markers on lymphoma cells, Karyotypes, etc were analyzed. 2) Among DLBCL cases, 76 cases treated with R-chemo were selected and analyzed in point of the response to treatment and overall survival. *Results.* 1) DLBCL 128, Follicular lymphoma 58, MALT 7, CLL 4 and so on. Among DLBCL cases, 6 cases (4%) were CD20 negative and three were positive by IHC. Weak expression were obsereved in 15 cases (B: 4, C:11). Among DLBCL, expression of CD20 were significantly weaker in CD5 positve group compared with CD5 negative group(P=0.01), age of negative group tend to be high (74.28 *vs.* 64.36, P=0.06, t-test) . However; Among thsese groups, in point of gender, IPI, clinical stage, biopsy lesion, expression of Karyotype statistical significance were not obsereved. Among FL cases, there were no CD20 negative, but low expression were obsereved in two cases(3.4%). 2)76 cases of DLBCL treated with R-Chemo were analyzed with Kaplan-Meier method. In the point of overall survival, GroupA(CD20negative) were significatlly lower. Between B+C(CD20 weak) and D(CD20 normal), there were no statistical significance. Some amount of CD20 may be enough to treat B cell lymphoma patient. *Summary and Conclutions.* Weaker expression of CD20 was observed in any types of B cell lymphoma. Among DLBCL, expression of CD5 and age are highly associated with weaker CD20. In DLBCL, weakness of CD20 may be the prognostic factor of DLBCL. Further analysis will be necessary.

1546

VIRAL INFECTIOUS IN CHRONIC LYMPHOPROLIFERATIVE DISORDERS - IMMUNOPHENOTYPIC CHANGES

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Background. Chronic lymphoproliferative disorders (CLD) frequently associate coinfection with hepatitis viruses, which may possibly be involved in their pathogeny. *Aim.* Immunophenotype changes of malignant lymphocyte in B-cell CLD with viral infections. *Materials and methods.* Bone marrow aspirate, peripheral blood samples on EDTA were available for analysis from 41 patients with CLD. Immunophenotyping was performed on a four color FACS Calibur according to EGIL/WHO panel. 13 CLD patients without viral coinfections were used as controls. Identification of B or T lineage of malignant cell was followed by detailed analysis of immunophenotype and clonality assessment. For statistical analysis we used SPSS software. *Results.* Disease type: 90% B-CLD; 5% T-CLD; 5% Hodgkin disease. Viral infections incidence: 58.53% HCV, 34.41% HBV, 2.43% HBV+HDV, 2.43% coinfection HCV+HDV, 2.43% triple viral infection HBV+HCV+HDV. We studied the immunophenotypical pattern (Figure 1) in patients with CLD with viral coinfection and we observed significant differences only in CLL group with viral infection: elevated expression of CD20 (Median level 90% compared to 39% in controls - patients without viral infection), of CD79b (Median 58% *vs.* 31% in controls), and of CD23 (Median 67% *vs.* 37% in controls). CD19 (Median 95% *vs.* 92% in controls) was unchanged in CLD with viruses (v-CLD). Higher expressions in v-CLD were observed for: CD38 (Median 49% *vs.* 24% in controls); antiapoptotic proteins were increased only in v-CLL group: bcl-2 (Median 46% *vs.* 5% in controls) and cyclin D1 (Median 11% *vs.* 0.5% in controls). No change in ZAP-70 expression: Median 59.5% *vs.* 59.1% in controls.

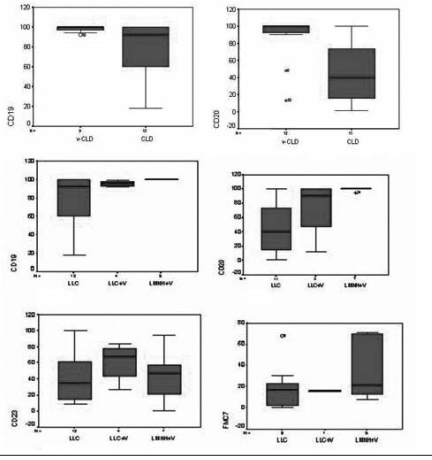

Figure 1. Boxplots representing different expression of CD20, CD79b, CD23, FMC7 in B-CLD with viral coinfection.

Conclusions. Hepatitis viruses could be involved in the pathogeny of CLD, but also as trigger for a more aggressive outcome. Higher expression of B-cell marker CD20, CD79b and CD23 suggest a lymphoma-like phenotype or a change to atypical CLL, associated with elevated expression of known prognosis markers bcl-2, cyclinD1, CD38. Unchanged expression of ZAP-70 could suggest a strong relationship with a basic unmutated IgVH status, and not with viral coinfection. Our interim data describe a significant change of immunophenotype of CLD in coinfection with hepatitis viruses, which suggest that the viral infection could produce a change to an atypical disease, with poor outcome.

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F-18 FDG PET AS A PREDICTOR OF CHEMOTHERAPY SENSITIVITY IN MALIGNANT LYMPHOMA

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1.The usefulness of the PET scanning for malignant lymphoma was discussed for many times and verified with the contribution to the diagnosis, treatment and recurrence evaluation. However, quantitative analyses were rarely identified for this disease entity. Although majority of the malignant lymphomas are chemotherapy sensitive tumors, only about 50% of the patients have long-term survival. High chemotherapy sensitivity may reflect that lymphoma has high proliferating tumor cells. 2.To evaluate quantitative value of FDG uptake as a proliferating index and clinical significance as a response predictor, we performed this study. 3.A retrospective analysis of 42 patients admitted to our institution for F-18 FDG-PET imaging before multiagent-chemotherapy was performed. Patients with a verified diagnosis of malignant lymphoma were included in this evaluation. In all study patients, reassessment of histological specimens was performed by a hematopathologist. In addition, the proliferating activity was analyzed using the Ki-67 (MIB-1) immunohistochemical assay and was judged by the percentage of lymphoma cells showing staining with the antibody. We investigated the nodal F-18-FDG uptake (visual analysis and semiquantitative analysis; SUV and relative uptake ratio) in lymphoma patient before chemotherapy. We compared maximum nodal F-18 FDG-PET uptake-ratio with Ki-67 expression that may be prognostically or therapeutically important, as well as with the response to multiagent chemotherapy. Attenuation-corrected whole body PET images were acquired 60 minutes after injection of 370-555 MBq FDG with a dedicated PET scanner (ECAT HR$^+$ scanner, Siemens-CTI, Knoxvile, Tenn., USA). Images visually interpreted by two experienced nuclear physicians, who had achieved consensus. We analyzed the degree of FDG uptake. Visually, the degree of FDG uptake was classified from grade -1 to grade 3: -1, lower; 0, equal; 1, slightly higher; 2, moderately higher; 3, intensely higher. Maximal standard uptake value (max SUV) and uptake ratio of max SUV for lesion to the mean SUV for contralateral basal lung were calculated. At diagnosis, median age was 49 years (range, 21-70). Hodgkin's lymphomas were in 6 cases. According to the WHO classifications, the most common histologic subtype was diffuse large cell lymphoma among 36 Non-Hodgkin's lymphoma patients. According to the Ann Arbor staging system, 4 patients had stage I disease, 21 patients had stage II disease, 12 patients had stage III disease, and 5 patients had stage IV disease. All chemotherapy regimens contained doxorubicin. 4. Nodal F-18-FDG uptakes were showed linear correlation with Ki-67 expression levels (correlation coefficient r=0.667, P=0.0001, Figure). Total response rate to chemotherapy was 70.2%. The patients with higher nodal F-18 FDG uptakes (grade +2, +3) had higher response rates than with lower nodal F-18-FDG uptakes (grade –1, 0, +1) (45.2% *vs.* 25.0%, P=0.018). The nodal F-18-FDG uptakes were significantly related with the responses to doxorubicin-based multiagent chemotherapy. 5. In conclusion, nodal F-18-FDG uptakes may be able to function as a predictor of chemotherapy sensitivity. Further studies for survival will be followed.

1548

GENE EXPRESSION PROFILING OF A SMALL COHORT OF FOLLICULAR LYMPHOMA PATIENTS FROM TUSCANY AND CORRELATION BETWEEN THE EXPRESSION OF ION CHANNELS AND LYMPHOMA GRADING

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Background. Non-Hodgkin's lymphomas (NHL) comprise a diverse group of diseases that are subclassified by the state of differentiation of the malignant B cells, presence of specific cytogenetic abnormalities, and characteristic morphology. Follicular lymphomas (FL) are the most common indolent NHL. Current diagnostic NHL categories do not adequately anticipate the tumor behaviour because there is considerable heterogeneity in tumors with the same diagnosis. This heterogeneity is often present in the tumor at the time of diagnosis and can be measured using microarray technique for gene expression profiling. *Aims.* The aim of this study is the characterization of the gene expression profile (GEP) of FL samples to better understand the biology of this disease and find new molecular targets that could be used in clinical therapy. *Methods.* We collected several primary lymph node tissues derived from Tuscany patients and we evaluated their GEP by whole genome microarrays. Among them, 7 FL samples were analyzed by bioinformatics tools and public databases. *Results.* We found that genes involved in clearly defined pathways were differentially expressed between lymphoma samples and normal lymph node tissue. In particular, we identified some genes of the NF-kB pathway, expressed by cells of tumor microenvironment implicated in the immune response and genes involved in the folate homeostasis. Moreover, using a specific clustering analysis focused on ion channels expression, we found an interesting evidence: the differential expression of ion channel genes reflects the molecular differences between different histological grades of the inspected samples, suggesting a correlation between such genes and FL grading. *Summary.* In this study we remarked the importance of microarray technology in understanding the molecular biology of diseases. In particular, in our samples, we identified some genes that are differentially expressed in FL patients and that are able to characterize distinct lymphoma grades, suggesting their different role in the lymphomagenesis and/or progression. This could be a starting point to evaluate new target therapy in this field.

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COX-2 EXPRESSION AND MICROVESSEL DENSITY IN DIFFUSE LARGE B CELL LYMPHOMA

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COX-2 and microvessel density are important factors in many solid tumors but there are no strong data for the DLBCL. Some studies showed that COX-2 expression and MVD are corallate by the poor prognosis. The role of the COX-2 in DLBCL pathogenesis remains a questionmark. Forty-nine formalin fixed, paraffin wax embedded DLBCL tissue samples from the archives of the clinics of pathology and the clinic of hematology at the University of Trakya were included in our study. The study made with the tissue micro array (TMA) construction and the area of 0.57 mm^2 stained with the CD34 and COX-2 (Cyclooxygenase-2) histochemistry slides were reviewed at magnification of x200. Expression of COX-2 and MVD (Micro vessel Density) were counted at the 10 binocular areas. MVD which was greater than 10, defined as elevated. COX-2 scored as 0 (negative), 1 (weak), 2 (medium) and 3 (strong) according to expression of the tissue. The tissues which were negative or weak and medium or strong COX-2 expression defined as negative and positive respectively. MVD, COX-2 expression, stage, treatment, overall survive are tested with Sapiro-Wilk test, Mann-Whitney U test, Chi-square and Chi-square Fisher Exact tests. COX-2 expression was greater in women (P=0.030). COX-2 expression was negative in 41 (83.7%) and positive in 8 (16.3%) patients. MVD counted at the range of 2-87. Median MVD reviewed as 12.5 (IQR=12.1). The patients whose COX-2 expression reviewed as negative and positive, MVD increased at 25 (61.0%) and 8 (100%) patients respectively. COX-2 expression was correlated with the MVD (P=0.031). There were no correlation between COX-2 expression and treatment response (χ^2=0.633; P=0.426). Median survival was determined as 12 weeks (OR 95% CI 8.95 - 15.05). There was no correlation between overall survive and the COX-2 expression (χ^2=0.175; P=0.676). No correlation has been found between overall survive and the MVD (χ^2=0.190; P=0.663). These data show that COX-2 expression and MVD are not correlate by the overall survive and treatment outcomes at diffuse large B cell lymphomas. In conclusion COX-2 expression and MVD are not increased in DLBCL and not correlate with the prognosis. More confirmatory studies are needed.

1550

BONE MARROW INFILTRATION IN MARGINAL ZONE B-CELL LYMPHOMAS

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Background. There are a various histological types of marginal zone lymphoma (MZL). Although all are thought that to have a common germinal centre origin, they present different clinical, treatment, and prognostic characteristics. *Aims.* The purpose of our study was to determine the relation between certain patterns of bone marrow infiltration and the

type of MZL. *Methods.* Here we analysed different patterns of infiltration in bone marrow biopsy from 50 MZL cases which presented bone marrow infiltration at diagnosis: 37 splenic MZL (SMZL), 7 nodal MZL (NMZL), and 6 mucosa-associated lymphoid tissue lymphoma (MALT). We differentiated 4 patterns of bone marrow infiltration (nodular, interstitial, diffuse, paratrabecular). We also took into consideration clinical and analytical parameters such as age, sex, ECOG, B2-microglobuline, number of nodal and extranodal areas, IPI, haemoglobin, leukocytes, neutrophils, lymphocytes, platelets, cariotype, and transformation to high-grade lymphoma. *Results.* We found that the most frequent pattern was nodular (48%), followed by interstitial (38%), diffuse and paratrabecular. When we compared bone marrow infiltration patterns according to MZL histological subtype we observed that in SMZL and MALT, the most frequent pattern was nodular, followed by interstitial and diffuse, while in NMZL, the most frequent pattern was interstitial (see table). However, none of the differences were statistically significant (P=0.28). An additional analysis was carried out which classified cased into only 2 groups of infiltration pattern (nodular and non-nodular). No significant correlation was found with the types of lymphoma under study (P=0.125). With respect to clinical and analytical parameters of each bone marrow infiltration pattern, the only statistically significant difference found was B2-microglobuline (P=0.046), which was higher in non-nodular patterns. *Conclusions.* 1. Our MZL cases presented a wide variety of infiltration patterns in bone marrow biopsy. 2. We could not demonstrate the existence of any infiltration pattern in bone marrow biopsy which is specifically related to MZL type, with the possible exception of the association between diffuse pattern and SZML. 3. It is possible that a nodular infiltration pattern reflects lower tumour activity, while diffuse patterns suggest high tumour activity. 4. A proper diagnosis of MZL type must take into consideration the combination of bone marrow infiltration, immunophenotype, and clinical parameters. Moreover, confirmation of MZL type should involve a biopsy from another nodal or extranodal organ whenever possible.

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THE USEFULNESS AND LIMITATIONS OF COMBINED FINE-NEEDLE ASPIRATION IMPEDANCEMETRY AND FLOW CYTOMETRY IN THE DIAGNOSIS AND SUBCLASSIFICATION OF NON HODGKIN'S LYMPHOMA

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Background. Cell morphology and immunophenotype are two primordial features in diagnosis and sub classification of non-Hodgkin`s lymphoma (NHL). The Coulter ACT Diff instrument gives an impedance size distribution histogram, which is very helpful in assessing lymphoid cells size. WBC histograms display the classification of leukocytes according to size following lysis. *Aims.* We reviewed our experience based on fine needle aspiration by combining impedancemetry and flow cytometry to determine their usefulness based on the new WHO-classification of malignant lymphoma. *Methods.* Impedance size distribution histograms and flow cytometry reports of patients who underwent both methods at the same time were examined. Both methods were classified according to the new WHO-classification of malignant lymphoma 2008. *Results.* There were total 50 cases included in this study. In seven cases FNA failed in gathering sufficient material for immunophenotyping. In ten cases there was no monoclonality B or T (5 Hodgkin's lymphomas, 3 carcinomas, and 2 lymphadenitis). 33 cases were diagnosed as NHL and were classified on impedancemetry as predominant small cells (19) and large cells (14) and on flow cytometry T NHL (4) and B NHL (29). Light chain restriction was demonstrated in 19/29 cases of B-NHL. With the help of combined impedancemetry and flow cytometry, it was possible to sub-classify 16 cases of the 33 NHL (48%) according to WHO classification. 17 cases were labelled as NHL not otherwise specified (NHL-NOS). *Conclusions.* Impedancemetry combined with flow cytometry may be helpful in accurately diagnosing and sub-classifying NHL into B or T types and small or large cell types. These methods are not sufficient to sub-classify all lymphomas according to WHO classification and the histopathology remains the main tool for the WHO classification.

1552

RADIOIMMUNOTHERAPY FOLLOWED BY BEAM CONDITIONING REGIMEN AND AUTOLOGOUS TRANSPLANTATION IS EFFECTIVE AND SAFE IN HIGH RISK RELAPSED/RESISTANT NON HODGKIN'S LYMPHOMA: A SINGLE INSTITUTION EXPERIENCE

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Background. High dose chemotherapy (HDC) followed by autologous stem cell transplantiation (ASCT) is an effective approach for the treatment of relapsed aggressive NHL. High risk patients i.e no response prior ASCT or early relapse have a dismal prognosis even with this approach with a 2-year PFS lower than 30-20%. Radiolabelled immunotherapy (RIT) with 90Yttrium Zevalin delivers targeted irradiation without TBI toxicity. Standard dose Zevalin (0.4 mCi/kg) combined with conventional conditioning regimen BEAM (Z-BEAM) was tested with promising results in the treatment of high risk relapsed/resistant aggressive NHL. *Aims.* We evaluated the efficacy as complete remission (CR) progression free survival (PFS) and overall survival (OS) of Z-BEAM followed by ASCT in a group of high risk relapsed/refractory patients treated in a single institution. The safety was considered as hematologic and extra-hematologic toxicity. Patients and methods Between October 2006 and October 2009 twenty patients were treated with Zevalin (day -14) followed by standard dose BEAM (day -7 to -1) and ASCT. Patients were considered at high risk of failure if: progression or early relapse (<1 year) after first line therapy, presence of multiple relapses or partial/no remission disease before Z-BEAM. Rituximab+ DHAP/ICE was used as induction and mobilizing treatment. *Results.* Clinical characteristics were: median age 47 years(range 27-69), eleven refractory and nine early or multiple relapse; fifteen grade III follicular/PML/DLBCL, three MCL and two indolent; five stage II and fifteen stage III-IV; nine had bulky disease and ten bone marrow involvement; ten had LDH level above normal. Eight patients received one previous therapy and twelve were resistant or relapsed after[3] 2 lines before Z-BEAM. All patients were previously treated with Rituximab. Response status before Z-BEAM was: CR 5 (25%), PR 8 (40%), SD/PD 7 (35%). At the end of treatment response status was: CR 12 (60%), PR 6 (30%) and SD/PD 2 (10%). Overall response rate was converted from 65% to 90% after Z-BEAM+ASCT. Z-BEAM was able to convert 4/7 patients with a SD/PG status in PR/CR at the end of therapy. With a median follow up of 25 months PFS and OS were 68% and 80% respectively. Two refractory pts before Z-BEAM showed a subsequent progression and four patients relapsed; four patients died for lymphoma 4,6,10,12 months after ASCT. Median $CD34^+$ cells infused was 7.26 106/kg (range 4.43-8.9). All patients engrafted with median time to platelet and neutrophils count higher than 20×10^9/L and 0.5×10^9/L of 11 and 10 days respectively. Febrile neutropenia occurred in twelve patients (60%), all resolved with empirical antibiotic therapy. No toxic deaths were recorded. Late toxicity (>100 days after ASCT) (lung Aspergillosis and cardiac failure) was recorded in only one patients heavily pre-treated with antraciclines and mediastinal radiotherapy. *Conclusions.* In this group of patients with high risk relapsed/resistant aggressive lymphoma Z-BEAM+ASCT was able to achieve a high response rate with complete engraftment and acceptable toxicity, not different from conventional BEAM. PFS appears to be improved compared to historical data. This approach needs to be tested in a larger multicenter study with longer follow up.

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CLINICAL IMPACT OF STAGING FOLLICULAR LYMPHOMA USING 18-FDG-PET/CT VERSUS DIAGNOSTIC CT

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Background. Follicular lymphomas (FL) are known to be 18-FDG avid. Integrated 18-FDG-PET/CT (PET/CT) could prove useful in FL due to the importance of more accurately discriminating local from disseminated disease. Reviewing the literature, there is a paucity of data comparing the clinical impact of staging FL with PET/CT *vs.* diagnostic CT. *Aims.* To evaluate the clinical impact of staging newly diagnosed or relapsed FL patients (pts) with PET/CT *vs.* diagnostic CT. *Methods.* Pts with biopsy proven FL who had been staged using PET/CT at diagnosis or relapse were included in this retrospective study. A lymph node was defined as pathological if FDG avid by qualitative visual assessment or if above the threshold size limit for CT criteria of the anatom-

ical region. Numbers of lymph nodes were counted on regional basis according to the manikin used in the FLIPI scoring index. Two experienced haematologists specializing in lymphoma treatment determined a management strategy based on diagnostic CT followed by a management strategy based on PET/CT. If radiation therapy was included, an oncologist reviewed the scans to determine if the radiation field was changed by the PET/CT compared to diagnostic CT. Changes in management strategy were defined as major (curative to palliative) or minor (changes in chemotherapy type/ number of cycles/radiation field). *Results.* Twenty-four newly diagnosed pts and four relapsed pts with FL were eligible (median age: 57). The FL's were characterized pathologically as grade I (9 pts), grade II (15 pts), grade III (two pts) and undefined (two pts). PET/CT identified 138 pathological lymph node regions whereas diagnostic CT identified 111. PET/CT detected additional pathological lymph node regions in 43 % of pts (95% CI 24-62). PET/CT identified CT unseen extra-nodal disease in three pts. PET/CT upstaged one patient (from II to III Ann Arbor) and upstaged FLIPI score in three pts. For CT stage I, PET/CT did not detect additional disease in any pts. However, in CT stage II-IV, PET/CT detected additional disease in a significant number of pts (Figure). PET/CT did not confer any major change in management strategy in any of the pts. However, in one patient, PET/CT did lead to a minor change by increasing the size of the radiation field against an extra-nodal area in a stage IV patient. *Conclusion.* PET/CT reveals more pathological lymph node regions in FL pts compared to diagnostic CT as it identifies disease in non-enlarged nodes. PET/CT is also better at detecting extra-nodal disease. However, we found no major clinical impact of performing PET/CT staging in FL patients compared to conventional staging. The role of PET/CT in FL needs to be defined in larger prospective studies.

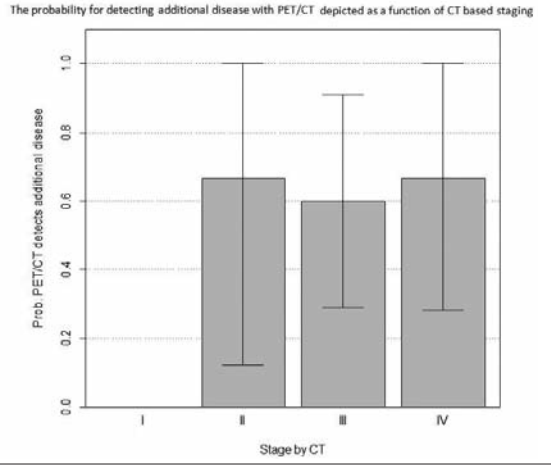

Figure.

1554

OCULAR ADNEXAL LYMPHOMA: A CLINICOPATHOLOGIC STUDY WITH NO EVIDENCE OF ASSOCIATION WITH CHLAMYDOPHILA PSITTACI IN OUR GEOGRAFICAL AREA

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Background. Ocular adnexal lymphomas (OAL) are a heterogeneous group of malignancies accounting for approximately 1-2% of non-Hodgkin lymphomas and 5-15% of extranodal lymphomas. OAL constitute the most frequent malignant tumor of the orbit. Analysis of somatic mutations in the V region of the Ig heavy (H) chain gene segment have suggested a role of chronic antigen stimulation. A variety of microbial pathogens that underlie chronic inflammatory processes can eventually lead to the acquisition of primary ocular adnexal MALT lymphoma (OAML) and may also play a pivotal role in both malignant transformation and subsequent clonal expansion of the lymphoma. OAML was initially associated with infection by *Chlamydophila psittaci*, although further reports did not support this association. Studies carried out using centralized molecular analyses have detected a certain geographical heterogeneity by showing discrepancies in the prevalence of *Chlamydophila psittaci* infection on OAML among countries and among different regions within the same country. *Aims of the study.* With this background, we decided to analyze the pathological and clinical characteristics in a series of 42 consecutive patients diagnosed with AOL in the same institution, to ascertain the pathogenic association with *Chlamydophila psittaci* in our area, and to detect the presence of t(11;18)(q21;q21) in OAML. *Methods.* Forty-two cases of OAL were enrolled in the study. The main clinical characteristics were recorded and analyzed. Pathological diagnosis was independently reviewed by two observers. DNA was extracted from the paraffin-embedded tissue samples and analyzed by PCR for IgH chain gene rearrangement and for *Chlamydophila psittaci* detection (n=28). In twenty-eight cases the translocations t(11;18)(q21;q21) was analyzed by fluorescence *in situ* hybridization. *Results.* Among 42 cases of AOL, 28 were diagnosed with OAML, 6 with DLBCL and 8 with other diagnosis (Table 1).

Table 1. Clinical features of patients with ocular adnexal lymphoma.

Characteristic	No. of patients OAL n=42 (%)	No. of patients OAML n=28 (%)
Age, y		
Median	71,5	70,4
Range	31-89	31-89
Sex		
No. female/no. male	21 / 21	16 / 12
Involved sites, no.		
Orbit	25 (60)	17 (61)
Lacrimal gland	7 (17)	4 (14)
Conjuntiva	5 (12)	5 (18)
Eyelip	3 (7)	1 (4)
Retroconal	2 (4)	1 (4)
Stage, no.		
I	29 (69)	22 (79)
II	3 (7)	2 (7)
III	2 (4)	
IV	8 (19)	4(14)
Histologic category, no.		
MALT	28 (67)	28 (100)
DLBCL	6 (15)	
Follicular	5 (12)	
Mantle cell	1 (2)	
Lymphoplasmacytoid	1 (2)	
Anaplasic T-cells	1 (2)	
Symptoms, no.		
Mass/swelling	26 (62)	17 (61)
Proptosis	12 (29)	8 (29)
Diplopia	3 (7)	2 (7)
Eye redness	1 (2)	1 (4)
Lateral, no.		
Left	23 (55)	14 (50)
Right	10 (24)	8 (29)
Bilateral	9 (21)	6 (21)

MALT denotes mucosa-associated lymphoid tissue
DLBCL denotes diffuse large B-cell lymhoma

The OALs were anatomically distributed as follows: orbit in 25 patients (60%), conjunctiva in 5 (12%), lacrimal gland in 7 (17%), eyelid in 3 (7%) and retroconal in 2 (5%). Bilateral involvement was observed in 9 patients (21%). Patients with OAML had a median age at diagnosis of 70 years (range, 31-89) and 57% were female. The most common presenting symptoms in OAML were mass/swelling in 17 patients (61%). No patient had B symptoms. The majority of patients with OAML (79%) presented at IE stage. IgH rearrangement was detected in 25 out of 28 cases with OAML, whereas the t(11;18)(q21;q21) was found in only 2 patients. PCR for *Chlamydophila psittaci* infection was negative in all 28 tumor specimens. After a median follow-up of 43 months, estimated 10-year overall survival was 71% (95% CI: 53%-99%) for OAL patients and 88% (95% CI: 70.5%-100%) for OAML. *Conclusion.* The great majority of patients with OAML presented as localized disease and indolent behavior. The lack of evidence of Chlamydophila psittaci infection in this series, in contrast to some prior reports, indicates that there may be geographic heterogeneity in the pathogenesis of OAML. Finally, the incidence of t(11;18)(q21;q21) was lower (7%) than previously reported in other locations (13.5%-30%).

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HERPESVIRIDAE VIRAL INFECTIONS FOLLOWING CHEMOTHERAPY IN PATIENTS WITH DIFFUSE LARGE B CELL LYMPHOMA UNDERGOING RITUXIMAB COMBINED CHEMOTHERAPY: INCIDENCE AND RISK FACTORS

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Background. Herpesviridae family includes herpes simplex virus, varicella zoster virus, Epstein-Barr virus, and cytomegalovirus, etc. Herpesviridae viral infection (HVI) can lead to serious complications including dissemination, secondary infection, bacterial super infection in patients with lymphoma undergoing chemotherapy. But there was no consensus on the dose and duration of antiviral agents prophylaxis in diffuse large B cell lymphoma undergoing Rituximab combined chemotherapy. We retrospectively analyzed the incidence and the risk factors for HVI. *Methods.* A total of 128 patients who newly diagnosed and received an chemotherapy with or without prophylaxis of acyclovir at the South Korea between Jan 2004 and Feb 2010 were enrolled retrospectively in the current study. HVI was confirmed based on clinical diagnosis, serologic test or pathologic diagnosis. The characteristics of the patients were as follows: the median age was 56years (range 22-81 years) with a female-to-male ratio of 59:69. The enrolled diseases included only diffuse large B cell lymphoma undergoing Rituximab combined chemotherapy. The results were analyzed using a chi-square test and independent samples T test. For the multivariate analysis, we used logistic regression test.

Figure 1. Curves for cumulative incidence of herpesviridae viral infection in all patients with diffuse large B cell lymphoma undergoing chemotherapy.

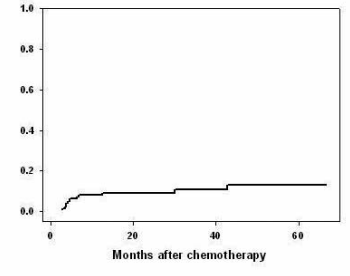

Figure 2. Curves for cumulative incidence of herpesviridae viral infection in patients with diffuse large B cell lymphoma undergoing chemotherapy. solid lines represent the more than one line, and dashed lines signify only one line of total number of chemotherapy and radiotherapy.

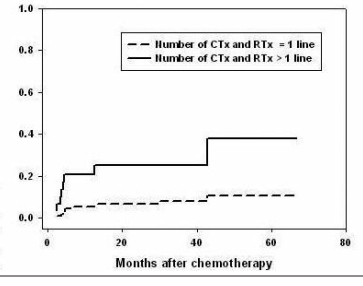

Figure.

Results. Thirteen patients (10.2%) developed HVI at a median of 9.96 months (range 2.53-42.90 months) after initial chemotherapy. In univariate analyses, risk factors for HVI were cumulative dose of steroid (P=0.018 , 6.3% in less than 4000mg *vs.* 21.9% in more than 4000mg), total number of sequential chemotherapy and radiotherapy (P=0.002, 5.1% in only one line *vs.* 27.6% in more than one line), presence of relapse (P=0.010, 27.3% in relapse *vs.* 6.6% in non-relapse). In multivariate analysis, the results confirmed only one variable as independent predictive factors for the total number of sequential chemotherapy and radiotherapy (P=0.032, hazard ratio (HR): 7.719, 95% confidence interval (CI) 1.189-50.096). There was no different mortality rate and survival rate between HVI and non-HVI group. *Conclusion.* Number of sequential chemotherapy and radiotherapy of more than one line was seemed to be high risk for HVI in patients with diffuse large B cell lymphoma undergoing Rituximab combined chemotherapy. Low-dose acyclovir prophylaxis for HVI may be needed in higher risk lymphoma patients undergoing chemotherapy.

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IS 18F-FDG-PET USEFUL IN FOLLICULAR LYMPHOMA? A PRELIMINARY REPORT FROM A SINGLE CENTRE EXPERIENCE

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Background. Follicular lymphoma (FL) is one of the most frequently occurring lymphoma entities in Europe and North America and is the most common indolent lymphoma. 18F-FDG-PET is becoming a routine measure for staging and follow-up of patients with aggressive lymphoma. By contrast, there are more limited data on its use in indolent lymphomas and, in particular, the role of this methodology in FL is still under investigation. *Aims.* We evaluated 18F-FDG-PET/CT (PET) at diagnosis and in the follow-up of patients with FL aiming to assess its possible usefulness on staging and clinical outcome. *Methods.* 18F-FDG-PET/CT (PET) was employed in all patients with FL referred to our Institution during the last three-year period. Overall, study population included 21 patients (11 males, 10 females; median age 58 years, range 42-77) with FL WHO grade I-II-III (5, 8 and 6 patients, respectively), plus 2 patients with mixed diffuse large B-cell lymphoma/FL. A total of 28 PET scans (9 before therapy and 19 for response assessment after treatment) were evaluated and compared with conventional staging. In 7 patients PET evaluation was longitudinally performed before and after therapy. Treatments were heterogeneous and given according to local policy, age and clinical conditions of patients, and research protocols applied: they included FND (Fludarabine, Mitoxantrone, Dexamethasone) with or without Rituximab, R-CHOP or R-CHOP- like regimens, R-CVP, R-FM (Rituximab, Fludarabine, Mitoxantrone). Two patients received Rituximab or Chlorambucil alone, respectively. *Results.* At diagnosis, seven cases demonstrated FDG avidity (78%), with a mean maximum standardized uptake value (SUV) of 8.2 (range 6.1-10.4). In one of the PET negative cases, disease had been completely excised. PET scan showed less diffused involvement than computed tomography (CT) in 2 of 9 patients, without, however, changing in the stage. At re-staging, twelve out of 19 evaluated patients had both CT and PET negative: all these patients maintain complete remission after a median follow-up of 38 months (range 21 to 56). By contrast, three patients who had CT negative/PET positive findings at re-staging experienced relapse/progression 12 to 23 months after completing induction therapy. Interestingly, one of these patients had achieved molecular remission after therapy, as showed by negative PCR for t (14;18) transcript. Four patients were PET and CT positive after therapy. All had progressive disease within few months and underwent alternative approaches. *Conclusions.* Though still preliminary and requiring confirmation on larger numbers of patients, our data indicate that FL is frequently FDG-avid and that persisting positive PET after treatment can contribute to identify patients with poorer prognosis.

1557

TREATING HIV-RELATED LYMPHOMA WITH CURATIVE INTENT DURING THE HAART ERA

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Background. Before the advent of effective antiretroviral therapy many HIV positive (pos) patients (pts) with Hodgkin (HL) and non Hodgkin lymphoma (NHL) couldn't receive adequate treatment due to poor clinical conditions or early deaths. The introduction of highly active antiretroviral therapy (HAART) in 1997 has reduced HIV-related complications, making HIV-related lymphoma (HIV-Ly) more treatable. Similar results as for HIV negative (neg) pts are reported for HIV-Ly who received aggressive treatment, at least in selected series. *Aims.* To evaluate the chance of HIV pos pts with HL and NHL to receive adequate treatment, before and after the advent of HAART. *Methods.* We evaluated the proportion of pts consecutively diagnosed with HIV-Ly at our center, who received treatment with curative intent, in the pre-HAART and HAART era, in comparison with our concomitant series of HIV neg pts. Pts were excluded from treatment in case of poor performance status, major infections or severe comorbidities. Pts aged more than 65 were excluded.

Results. From 1985-1996 (pre-HAART period), we diagnosed 109 HIV-Ly, 84 systemic aggressive NHL (sNHL), 14 primary central nervous system lymphoma (PCNSL) and 11 HL and from 1997-2008 (HAART period) 102 HIV-Ly, 67 sNHL, 6 PCNSL and 29 HL. Before 1997, we could treat with curative intent 67% pts with sNHL [complete remission (CR) 56%] and 21% PCNSL. After 1997, the proportion increased to 78% for sNHL (P=0.1) (CR 57%), while only 1 pt with PCNSL received treatment. In the HAART era only 55% of pts were on HAART at sNHL diagnosis. However, the proportion of treated pts was similar between pts receiving or not HAART (76% *vs.* 80%). Only 1 pt was on HAART at PSNCL diagnosis. The long-term OS of treated pts in the HAART era was significantly better compared to the pre HAART period (52% *vs.*14%, P=0.0006). Within our series of HIV-related HL, we could treat 64% of pts in the pre-HAART (CR 43%) and 72% in the HAART era (CR 62%). After 1997, 65% of pts were on HAART at HL diagnosis; 79% of them received treatment, compared with 62% of those who started HAART at diagnosis. The long-term OS of pts treated after 1997 wasn't significantly better than before (35% *vs.* 29%). Within our consecutive HIV neg pts with lymphoma aged less than 66, we treated after 1997 96.3% sNHL, (compared to 78% of HIV pos pts, P<0.0001), and 100% of PCNSL (compared to 17%; P=0.004). Concomitantly, all (100%) HIV neg pts with HL, younger than 65, received full-dose treatment at our center. *Conclusions.* In our single center series of consecutive non selected HIV-Ly, the proportion of pts who cannot receive adequate treatment, due to poor performance status or comorbidities, remains significantly higher compared to the HIV neg population, even in the HAART era. The outcome of treated is significantly improved, at least for sNHL. Treating PCNSL in HIV pos pts remains a challenge even in the HAART era.

1558

CLINICAL IMPACT OF BULKY MASS IN THE PATIENT WITH PRIMARY EXTRANODAL DIFFUSE LARGE B CELL LYMPHOMA TREATED WITH R-CHOP THERAPY

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Background. Although numerous studies about primary extranodal diffuse large B cell lymphoma (DLBCL) were reported sporadically, the literature of clinical value of immunophenotype and bulky diameter in rituximab era is limited. *Aims.* We analyzed in primary extranodal DLBCL patients treated with R-CHOP (rutiximab, cyclophosphamide, doxorubicin, vincristine and prednisone) therapy to evaluate whether immunophenotype and size of bulky disease are significantly important. *Patients and methods.* A total of 96 patients with primary extranodal DLBCL were enrolled in the present study. All patients were treated with R-CHOP therapy. Radiotherapy at extranodal site was given to 10 patients by physician's discretion.

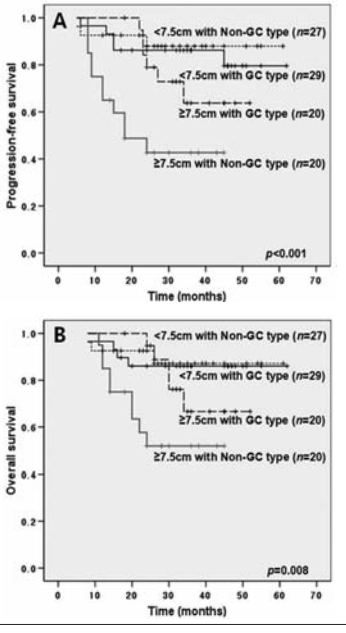

Figure.

Results. A total of 96 primary extranodal DLBCL patients were analyzed in the present study. Median follow-up duration was 36 months. Ann Arbor stage at diagnosis were IE (n=23) and IIE (n=73). Forty-four patients had an elevated LDH status at the time of diagnosis. Fifty-one patients were >60 years of age and 36 subjects had an Eastern Cooperative Oncology Group (ECOG) performance status >2. The GC type was applied to 49 of 96 cases; the other 47 were of the non-GC type-International prognostic index (IPI) was still important prognostic factor for progression-free survival (PFS) and ov! erall survival (OS) (P=0.003, P=0.027). Difference of survival between immunohistochemically germinal center (GC) type and non-GC tpye was not different (PFS, P=0.192; OS, P=0.197). In two separated groups according to extranodal maximum tumor diameter (EN-MTD) 7.5 cm as cut-off value for survival, the group of EN-MTD ≥7.5 cm had lower PFS and OS than <7.5 cm (PFS, P=0.001; OS, P=0.008). In four divided subgroups according to EN-MTD combined with immunophenotype, the subgroup of non-GC type with EN-MTD ≥7.5 cm had lower PFS and OS compared with other subgroups (PFS, P<0.001; OS, P=0.008). Multivariate analysis revealed that non-GC with EN-MTD ≥ 7.5cm was an independent prognostic parameter (PFS, HR=5.407, 95%CI=2! .378-12.294, P<0.001; OS, HR=4.136, 95%CI=1.721-9.941, P=0.002). *Conclusion.* It appears that bulky primary extranodal DLBCL would be associated with unfavorable outcome especially in non-GC type. More intensive treatment to the patients may improve the outcome.

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IMMUNE RECONSTITUTION IN PATIENTS WIHT B-NON HODGKIN LYMPHOMA (B-NHL) TREATED WITH RITUXIMAB, CHEMOTHERAPY OR BOTH - A COHORT STUDY

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Background. B-NHL patients present prolonged periods of myelo and immunosuppression after (immuno)chemotherapy, leading to an increased risk of infections and possibly tumor recurrence. *Aims.* We prospectively analyzed the immune reconstitution kinetics and profile of infections in B-NHL patients treated with Rituximab (R), Rituximab+Chemotherapy (RCT) or Chemotherapy only (CT). *Methods.* Clinical and immunological characterization (complete blood counts, lymphocyte subpopulations, immunoglobulin serum levels and analysis of medical records), were done before and up to 7 months (M) after treatment. The study was approved by the local Ethics and Research Committees; all patients signed informed consent. *Results.* Thirteen patients (69% men, median age of 69, 47-86 yo), including 7 CLL, 2 MALT, 2 B-NOS, 1 Lymphoplasmocytic and 1 Follicular received CT (54%-Fludarabine/Cyclophosphamide, 38%-Chlorambucil/Prednisolone and 8%-Fludarabine/Mitoxantrone/Cyclophosphamide); 28 patients (38%-men, median age 63.5, 44-82 yo) received RCT (68%-CHOP, 28%-RCVP, 4%-RCHOP and RCNOP), including 10 Follicular, 10 DLBCL, 3 MCL, 1 MALT, 3 B-NOS and 1 Lymphoplasmocytic; 13 MALT patients (62%-men, median age 65, 43-82 yo) received R. All patients under R or RCT significantly decrease peripheral blood B-cell numbers 1 and 4M after treatment, compared to pre-treatment, P=0.009 and P=0.01, respectively. However, patients receiving R showed higher B-cell numbers 7M pos-treatment when compared to RCT patients (3.5 *vs.* 0.5 fold increase at 1 and 7M, respectively). IgM levels were most affected by treatment. RCT patients have a significant decrease of serum IgM and IgG, 1 and 4M after-treatment compared to pre-treatment (P=0.029, P=0.026, for IgM and P=0.002, P=0,036 for IgG, respectively). Patients receiving R or CT showed no significant alterations in Ig levels during follow-up. $CD3^+$T-cells were reduced by all treatments, and significantly by CT regimen at 7M pos-treatment (P=0,039). Only patients receiving CT alone (mostly with purine analogs) revealed a significant decrease of $CD4^+$ and $CD8^+$T-cells subpopulations at all time points compared to baseline values (P=0.006). $CD4^+$ naïve T-cells ($CD4^+CD45RA^+CD62L^+$) suffered a significant decrease compared to $CD8^+$ naïve T-cells ($CD8^+CD45RA^+CD27^-$) in patients treated with RCT (P=0.001): 50% *vs.* 13% from pre-treatment to 1M pos-treatment. The same regimen significantly increased $CD4^+$ memory T-cells ($CD4^+CD45RA^-CD62L^-$) at 7M pos-treatment (1 to 1.4 increments, P=0.01) and decreased of $CD8^+$ memory T-cells ($CD8^+CD45RA^-CD27^+$) immediately (1M) after therapy. These may explain the higher incidence (4 to 11 times) of infections observed in this group of patients as compared to CT and R patients. In fact, bacterial, viral and fungal infections were more frequent during RCT treatment; those diminished during follow-up, although a cluster of fungal infections was again observed at 7M. Moreover, the frequency of infections was inversely and significantly correlated with %CD4+ naïve T-cells (P<0.001). *Summary/Conclusions.* Rituximab treatment does

not seem to significantly alter the immunological parameters including Ig levels and T-cells subpopulations. Also, B-cell numbers recovered faster in Rituximab only treated patients as compared to patients undergoing other treatments. This may explain the lower incidence and severity of infections observed. Naïve $CD4^+$T-cells seem to be relevant for protection against infections during treatment and short-term follow-up time.

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PROGNOSTIC SIGNIFICANCE OF LYMPHOMA ASSOCIATED MACROPHAGES (LAM), KI-67 AND HYSTOLOGICAL GRADE IN NEWLY DIAGNOSED FOLLICULAR LYMPHOMA PATIENTS

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Background. In spite of great progress in treatment of follicular lymphoma in past few years some patients still have bad clinical course of the disease and short survival. Many prognostic models have been suggested to identify this subgroup of patients, but they are exclusively based on clinicial and laboratory findings. The investigations of molecular hallmarks of lymphomas and their possible prognostic influence have been attractive for the past few years. *Aims.* The aim of this study was to investigate possible influence on survival of the number of CD68 positive cells (Lymphoma associated macrophages, LAM) in tumor tissue, proliferation index Ki-67 and hystological grade. *Methods.* In this retrospective study were included 50 newly diagnosed FL pts treated at the Clinic for Hematology Clinical Centre of Serbia from September 2001 until March 2006. The diagnose of FL in patients included in this study was made from lymph node biospy. The analysis was performed on specimen from paraffin embeded tissue of lymph node from diagnosis. *Results.* The patients with more than 10 intrafollicular LAM had signifficantly worse outcome than the patients who didn't have them (log-rank, P=0.018). There was no difference on survival regarding extrafollicular number of LAM. Patients with Ki-67>30% had signifficantly worse outcome compared to patients who had low Ki-67 (log-rank, P=0.002). There was no signifficant difference in survival of patients regrading to hystologic grade of follicular lymphoma, but the patient with grade 3 had tendency towards worse survival compared to patients with grade 1 and 2. In univariate analysis survival was influenced by number of intrafollicular LAM>10 (P=0.023) and Ki-67>30% (P=0.006). In multivariate Cox model, only Ki-67>30% was predictive for poor outcome (P=0.014). *Conclusion.* In our study patients with high number of intrafollicular LAM and Ki-67>30% had poor outcome, but Cox regression multivariate analysis identified only Ki-67 as independent variable. Regardless of this, we think that large multicenter study with unique hystopathological methodology is needed to estimate the influence of microenvironment, proliferation index and hystological grade on outcome and if it proves to have an influence, to be assesed for possible insertion in prognostic indices.

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OUTCOME AND PROGNOSTIC FACTORS IN FOLLICULAR LYMPHOMA. RESULTS WITH CHLORAMBUCIL WITH OR WITHOUT RITUXIMAB

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Background. Follicular Lymphoma (FL) is the most common low grade lymphoma. Due to its long natural history, prolonged follow-up is needed to evaluate the impact of different prognostic parameters. *Aims.* To analyze prognostic factors in a series of 118 FL patients diagnosed, treated and followed in a single Hematology Unit between 2000 and 2009 and to compare outcome between patients treated with or without Rituximab (R). *Methods.* 118 consecutive patients were retrospectively reviewed. The following parameters were examined by univariate analysis as possible prognostic factors: age, gender, performance status (PS), FLIPI score, clinical stage (CS), B symptoms, LDH, hemoglobin (Hb), absolute lymphocyte counts (ALC) and bulky disease. *Results.* 75% had grade 1 or 2 histology, 64% were males with a median age of 57.5 years (range 25-85). The distribution of CS was as follows: 30%, 12%, 15% and 44% for CS I, II, III and IV, respectively, while 12% had B symptoms and 91% a PS 0-1. Hb was <12g/dL in 18% and LDH was elevated in 13%. ALC were $<1.5\times10^9$/L and $<1\times10^9$/L in 13% and 39% of patients respectively. Bulky disease was evident in 25%, while 37% and 17% had 1 and ≥2 extranodal sites. Bone marrow involvement was evident in 42%. FLIPI score was 0-1 in 47%, 2-3 in 40% and 4-5 in 13%. The most frequent treatments were chlorambucil in 23 patients, R-chlorambucil in 40, R-CHOP in 25 and radiotherapy in 18. At a median follow-up of 47 months (7-114) the 5-year progression free survival (PFS) and overall survival (OS) were 59% and 89% respectively and did not differ among grades 1-2 and 3. However there was a trend for a plateau in the survival curve for grade 3 cases. The only significant prognostic factor for PFS was bulky disease (5-year PFS: 71% *vs.* 22%, P<0.001). FLIPI, PS, CS, ALC and bulky disease proved to be significant factors for OS. Patients with FLIPI score 0-1, 2-3 and 4-5 had a 5-year OS of 100%, 86% and 43% respectively (P<0.05). Patients with PS 2-4 had an inferior 5-year OS compared to PS 0-1 (80% *vs.* 91%, P=0.045). Limited CS (I-II) had a superior OS compared to advanced (III-IV) FL patients (100% *vs.* 85%, P=0.05). Low ALC were poor prognostic factors for OS at both cut-off levels (5-year OS: 91% for ALC$\geq1.5\times10^9$/L and $\geq1\times10^9$/L *vs.* 87% and 75% for ALC$<1.5\times10^9$/L and $<1\times10^9$/L respectively, P=0.045 and P=0.025). Patients with bulky disease had an inferior OS (70% *vs.* 98%, P=0.018).The impact of Rituximab was evident in the prolongation of PFS: Patients treated with R-chlorambucil had a superior 5-year PFS (78%) compared to those treated with chlorambucil only (40%, P<0.02). However OS did not differ between the two groups, most likely due to subsequent R-containing chemotherapy. *Conclusions.* In this retrospective analysis the FLIPI score, PS, CS, ALC and bulky disease proved significant prognostic factors, whereas histologic grade did not have any impact on OS. Moreover, the addition of RItuximab to chlorambucil prolonged PFS significantly compared to chlorambucil monotherapy.

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CONTINUATIVE IMMUNO-CHEMOTHERAPY FOLLOWED BY HIGH-DOSE AND AUTOLOGOUS CELL TRANSPLANTATION IN UNTREATED MANTLE CELL LYMPHOMA PATIENTS. EXPERIENCE AT THE EUROPEAN INSTITUTE OF ONCOLOGY IN MILAN

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Background. High-dose chemotherapy followed by autologous stem cells transplantation (ASCT) is considered a reasonable option in patients with mantle cell lymphoma (MCL) responding to anthracycline-based chemotherapy regimens. MCL cells express CD20 in their surface, providing a target for immunotherapy with Rituximab and even if the full impact of adding the anti-CD20 monoclonal antibody to the treatment of MCL remains unclear, it may be important. *Aims.* We analysed our experience with the use of Rituximab as *in vivo* purging in association chemotherapy followed by ASCT as front-line treatment in naïve MCL patients followed at our Institution from 2000. *Methods.* From May 2000 to August 2008 27 newly diagnosed patients with histological proven MCL were treated in our Institution. We considered 20 males and 7 female with MCL, with median age of 55 years old (range 39-66) and in advanced disease (stage III-IV 25/27). Twelve patients presented extranodal disease and 9 of them gastrointestinal involvement. Induction treatment included anthracycline-containing chemotherapy (ACOD) in combination with monoclonal antibody, followed by two cycles of DHAP-modified regimen (ESHAP) with Rituximab. Responding patients received a conditioning regimen (high-dose Melphalan plus anthracycline) in association with Rituximab at day -7 and followed by autologous stem cells reinfusion. All patients underwent restaging before and just after ASCT procedure. *Results.* Before transplantation 26/27 patients presented a clinical response, 19 were in complete response and 7 in partial. Three patients did not underwent ASCT, one because serious infection in course of induction CHT, another because of patients refuse and the last because of progressive disease. After ASCT 23/24 patients achieved a complete response. The median time to recovery neutrophils ($>0.5\times10^3$) was 11 days (range 5-20) and that of platelets ($>20\times10^3$) was 15 days (range 9-48). During aplasia, 22 patients developed fever, mainly (17/22) of unknown origin. No toxic death was observed. With a median follow up of 40 months (range 4-97 months) 17 patients are still alive and 12 of them in complete remission. *Summary/Conclusions.* According to other published experience, our experience seems to confirm that high-dose chemotherapy fol-

lowed by ASCT is a valid option as up-front treatment in MCL patients and the addition of Rituximab may play an important role in improving results of chemotherapy used alone, probably because of its *in vivo* purging and synergic effect.

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TREATMENT OF PRIMARY MEDIASTINAL B CELL LYMPHOMA (PMBCL) WITH RITUXIMAB-CHOP PLUS RADIOTHERAPY: A MULTICENTRIC EXPERIENCE

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Background. Rituximab-CHOP (R-CHOP) immunochemotherapy is the current standard treatment of diffuse large B-cell lymphoma, but its impact on PMBCL is being evaluated. *Aims.* To retrospectively evaluate clinical characteristics and outcome of patients with PMBCL treated with R-CHOP plus radiotherapy (RT) in three Italian institutions. *Methods.* Between 3/2003 and 5/2009, 31 untreated patients with PMBCL have been admitted in our institutions. Disease evaluation was performed with whole-body computed tomography at diagnosis, after 3-4 courses of chemotherapy and at the completion of immunochemotherapy. Finally, all patients received PET/CT after treatment conclusion. The planned treatment consisted of 6-8 courses of R-CHOP21 or R-CHOP14 plus involved field (IF) RT (25-44 Gy). According to our treatment strategy, patients who obtained at least a partial remission (PR) at first restaging completed planned therapy, while patients who failed to achieve complete remission (CR) or had progressive disease (PD) started high dose chemotherapy (HDC) followed by autologous stem cell transplantation (ASCT). Retrospectively, we evaluated the response rate, overall survival (OS) and freedom from treatment failure (FFTF). *Results.* Seven patients were male and 24 female, with a median age of 35 years (15-81). Stage I-II was present in 25 patients (81%) and stage III-IV in 6 (19%). Nineteen patients (61%) had B symptoms; aaIPI was 0-1 in 23 cases (74%) and 2-3 in 8 cases (26%). Bulky disease, defined as a mass of 10 cm or more in greatest dimension, was reported in 24 cases (77%).Twenty-nine patients completed R-CHOP program and 28 of them received IFRT. RT was omitted in an 81 year old patient with non-bulky IA clinical stage, who gained CR after ICHT. After R-CHOP with or without RT, 26 patients (84%) achieved CR, 2 patients (10%) PR and one (6%) progressed. The last 3 patients and 2 patients who were unresponsive during immunochemotherapy received salvage treatment with HDC and ASCT. Four of them achieved CR and one died of PD. Among the 30 patients who obtained CR following first-line or salvage treatment, none relapsed so far. After a median follow-up of 31 months, OS and FFTF were 96% and 84%, respectively. *Discussion.* The best therapy for PMBCL is unknown. In retrospective analyses, but not in prospective randomized studies, third generation chemotherapy regimens, namely MACOP-B or VACOP-B, plus RT achieved better results in comparison with conventional CHOP. We think that R-CHOP plus IFRT is feasible, safe and effective in inducing durable CR in a high proportion of patients with PMBCL. Moreover, HDC and ASCT seems an efficacious salvage treatment for patients with refractory PMBCL and this approach should be applied immediately at the first signs of unresponsive disease.

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CENTRAL NERVOUS SYSTEM INVOLVEMENT IN PRIMARY MEDIASTINAL LARGE B-CELL LYMPHOMA: A SINGLE INSTITUTION EXPERIENCE

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Introduction. Primary mediastinal (thymic) large B-cell lymphoma (PMLBCL) is recognized as subtype of aggressive non-Hodgkin lymphoma with unique clinical and immunopathologic characteristics and relatively favorable outcome. PMLBCL has a high propensity for involving distant extranodal sites including central nervous system (CNS). The aim of our study was to evaluate the frequency and pattern of CNS involvement in PMLCL. *Methods.* Beetween 2004 and 2009, 86 previously untreated patients (pts) with PMLBCL were diagnosed and treated at our center. The median age was 30 years (range 15-63) and 49\86 (56%) were females. Stage II/IIE (63%) resulting from direct extension of the mediastinal mass has 54 pts (63%). Patients often presented with B symptoms (75%), bulky mediastinal masses (73%), extranodal involvement (80%) and increased LDH (84%). All patients received a regimen containing doxorubicin (CHOP+R, MACOP-B+R) with or without RT as initial therapy. *Results.* Five patients (5,8%) developed brain parenchymal involvement, 3 pts during or very shortly (2 months) after completion of treatment, 2 pts at relapse. Increased LDG, B symptoms and extranodal disease occurred at presentation in all pts, 3 pts had distant sites involvement (kidneys, adrenals, ovaries, pancreas, stomach). According to the age-adjusted International Prognostic Index score (aaIPI) 4 pts had aaIPI=2-3. As initial treatment, two pts received R-CHOP, 1- CHOP, 2 - R-MACOP-B. Four pts died of CNS disease, one patient now is alive after MCP regimen (HD MTX on days 1, 15, 30, CCNU on day 1, procarbazine on days 1-10). *Conclusions.* These data demonstrate that all patients PMBCL with CNS involvement initially belonged to the high-risk group. CNS relapse occurs early (median time to relapse 2 months) and is associated with a very poor prognosis. The propensity to involve the CNS parenchyma raises the concern that intrathecal prophylaxis may not be effective, moreover 2 pts had systemic therapy incorporating middle-dose MTX (400 mg/m^2). Our data suggests that CNS imaging should be considered in patients PMBCL with extranodal disease and addition of high-dose MTX (>1g\m^2) to the primary therapy may reduce CNS recurrence.

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PROGNOSTICAL SIGNIFICANCE OF KI-67 EXPRESSION AND IPI SCORE IN PATIENTS WITH PRIMARY MEDIASTINAL LARGE B-CELL LYMPHOMA: A SINGLE CENTRE RETROSPECTIVE ANALYSIS

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Background. Primary mediastinal large B-cell lymphoma (PMBCL) is a relatively rare subtype of diffuse large B-cell lymphoma with distinctive clinical, immunophenotypic and genotypic features. In the absence of randomized trials, the prognostic factors and the optimal treatment of the patients with PMBCL remain a matter of debate. *Aim.* The aim of our study was to make a retrospective analysis of clinical features, prognostic factors, treatment approaches and outcome in patients with PMBCL. *Patients and methods.* Our study included 42 PMBCL pts who were treated in our institution between June 1999 and November 2009. Five patients were treated with chemotherapy (CT) including MACOP-B, EPOCH or CHOP and 37 pts with immunochemotherapy (ICT) (Rituximab+CT). Two patients had surgical resection of lungs followed with ICT. Twenty-eight patients (67%) received mediastinal involved-field radiotherapy (RT) at a median dose of 36Gy. All samples of lymph-node biopsies were analyzed for the immunohistochemical expression of Ki-67. The median follow up was 59 months. *Results.* The median age was 32±12,09 (range 19-73); 29/13 (69%) were females; 32 pts (76%) were younger than 40 yrs; 2 pts had stage I, 30 stage II, 7 stage III, 3 stage IV; 33 pts (78%) presented as a bulky disease; EN presentation had 8 pts (19%); Ki-67 expression ≥80% had 31 pts (74%); LDH was increased in 33 pts (78%), B2M in 23 (55%), and 19 (45%) had a superior vena cava syndrome. According to the IPI score, 22 pts had an IPI=0-1 and 20 an IPI=2-3-4. The response rate was CR/CRu= 31 pts (74%), PR=9 (21%) and NR=2 (5%). Twelve patients (27%) with PR achieved a CR after RT. Relapse of disease was found in 13 pts (31%) while 2 pts had two relapses. The 4-year OS and PFS were 61% and 52% respectively. The overall survival was significantly higher in female pts (log rank P=0,036), in pts with ECOG performance status <2 (log rank P=0,001) and in pts who received ICT (Tarone-Ware P=0,048). The patients in advanced CS (log rank P=0,04) had significantly shorter PFS. Analyzing the expression of KI-67 (<80% *vs.* ≥80%) and IPI score (<2 *vs.* ≥2), we found that these factors had the greatest prediction of disease progression. The patients with high Ki-67 expression had shorter PFS and frequent relapse of disease even in patients treated with ICT. *Conclusions.* Although the number of patients in the study was limited, data showed that the Ki-67 expression and IPI score have prognostic value in PMBCL patients. Futher more, the study showed that immunochemotherapy truely improves the survival and consolidation radiotherapy improves the quality of response in PMBCL patients.

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MALT VERSUS NON-MALT GASTROINTESTINAL (GIT) LYMPHOMAS-PROGNOSTIC PARAMETERS AND SURVIVAL

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Background. Most frequent lymphomas located in the digestive tract are low grade B cell lymphomas arising from mucosa associated lymphoid tissue (MALT) and diffuse large B cell lymphomas (DLBCL). The most common site of involvement is the stomach followed by the small intestine and the colorectum. *Aim.* This study compared patients with extranodal lymphomas based on pathohistological finding (MALT *vs.* non-MALT) as well as regarding gastric and non-gastric localization, and also determining the significance of clinical-laboratory parameters related to therapeutic response and length of survival. *Methods.* A total of 82 patients with extranodal non-Hodgkin's lymphomas of the gastrointestinal tract were evaluated over a period 1998./2008. We analyzed demographic features, clinico-bilogical parameters, outcome to therapy and survival. *Results.* Regarding the localisation of the disease, the stomach was most frequently affected (55 patients, 67.1%) followed by small and large intestines (27 patients, 32.9% patients). As for the pathohistological findings, MALT lymphoma accounted for 55%, DLBCL 37%, while other subtypes accounted for 8% patients. Patients with MALT lymphoma had Helicobacter pylori infection significantly more than patients with other type of lymphomas (P<0.001). Statistically significant difference in patients' distribution was not found with respect to IPI index, bone marrow infiltration, anemia, hypoalbuminemia, or histological subtype and localization. Fifty one patients received chemotherapy, while others received immunochemotherapy. Complete remission achived 53 patients. Five years overall survival was 60%. There were no differences in outcome of patients regarding to pathohistological type, affected gastrointestinal tract organ and type of therapy. Statistically significant differences according to survival probability was obtained based on sex (survival was longer in woman), according to CS regarding to Ann Arbor and Lugano classifications (the patients with lower CS live significantly longer); according to IPI index (the survival is significantly longer in patients with low IPI score 0,1, and 2), as well as patients free of bone marrow infiltration whose survival is also significantly longer. In univariate analysis Ann Arbor stage IV (P=0.001), Lugano stage IV (P=0,002), bone marrow infiltration (P=0.002), IPI score more than 2 (P=0.001) and elevated LDH level (P=0.055) were significantly associated with poor survival. But, in multivariate analysis only Ann Arbor stage IV (P=0.023) retained their prognostic significance. *Conclusion.* Our results did not confirm differences between pathohistological type, affected gastrointestinal tract organ and type of therapy regarding to survival. Also, the parameters of disease extension at presentation (CS, IPI, marrow infiltration) are the most important prognostic factor in our group of GIT lymphoma.

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THE ROLE OF FDG-PET SCAN IN PATIENTS WITH INDOLENT NON HODGKIN LYMPHOMA (NHL)

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Background. Positron emission tomography (PET) imaging using 18-fluoro-2-deoxyglucose (FDG) is routinely used in the management of Lymphomas. Although several studies have demonstrated PET sensitivity and specificity in high grade non-Hodgkin lymphoma (NHL) and in Hodgkin lymphoma, the intensity of FDG uptake is generally low in indolent lymphomas and few data are available regarding PET accuracy in low grade NHL . Several studies have concluded that PET appears useful for the staging of Follicular lymphoma (FL) but has a limited usefulness in the other indolent lymphoma subtypes. Aim of our study was to investigate the potential impact of PET in the management of patients with indolent lymphomas. *Methods.* We performed a retrospective single institution study on a population of 48 patients (pts) with indolent NHL, observed from September 2005 to January 2009, who underwent PET for evaluation of treatment response; 18 (37%) of them had also undergone PET at the time of the initial diagnosis. Median age was 56ys (37-86), 32 (66%) had stage IV, 15 (31%) LDH and 23 (48%) high β2μ globulin levels. Histology was: follicular lymphoma (FL) in 31 pts (65%), Marginal zone lymphoma (MZL) in 12 (26%), small lymphocytic lymphoma (SLL) in 3 (6%), Lymphoplasmocytic lymphoma in 2 (3%). All pts underwent CT scan, Bone Marrow biopsy, and physical examinations. In 16 (92%) pts PET was positive; 13 (75%) of them had FL. In 15 (85%) pts there was agreement between CT scan and PET. In 3, PET was positive and CT negative. Chemo-immunotherapy was administered in all pts, RCHOP like in 37 (77%), RFND 11 (23%). We performed a correlation between positive PET at diagnosis and the achievement of Complete Remission (CR). *Results.* Response to treatment was evaluated with CT scan, PET and clinical examinations. Thirty-four (70%) pts achieved CR, 14 (30%) Partial Remission (PR). In 27 pts (57%) there was agreement between CT scan and PET. In 16 (34%) pts PET was negative and CT positive. All these pts received more treatment and subsequently achieved CR. As to the correlation between PET at diagnosis and the achievement of CR: 14 (85%) pts with positive PET did not obtain CR. After a median follow up of 21 months (7- 57), comparing the two groups with negative and positive PET, respectively, at the end of treatment, Progression Free Survival (PFS) of pts with a negative PET was significantly better (P= 0.015). *Conclusions.* In our study, in agreement with literature, we observed that PET has a good sensitivity in FL; PET positivity at the initial staging is correlated with a worse response rate, and PFS at 2 years for pts with a negative PET at the end of treatment is significantly better than with a positive PET. Further studies need to be designed to investigate the role of PET in indolent lymphomas, the optimal timing for PET, and to establish a possible predictive value of PET for response to treatment and PFS.

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NT-PROBNP IS AN INDEPENDENT BIOMARKER OF MAJOR CHEMOTHERAPY-RELATED TOXICITY AND CLINICAL OUTCOME IN NON-HODGKIN LYMPHOMA

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Introduction. NT-ProBNP provides diagnostic and prognostic information in many heart syndromes but its role in lymphoma patients has yet not been established. The aim of the study was to investigate the association between NT-ProBNP levels and the risk of major chemotherapy-related toxicity and death. *Patients and methods.* We studied 104 consecutive patients with non-Hodgkin lymphoma treated with chemotherapy. High-resolution echocardiography and serum NT-ProBNP concentration were determined prior to chemotherapy. Major toxicity events after first-line chemotherapy and death from any cause were the primary end points of the study. An independent series of patients served as validation set. *Results.* With a median follow up of 13 months, 26 patients had a major toxic event related to first-line chemotherapy and 18 had died at last follow-up. The NT-ProBNP threshold with the best predictive accuracy for major chemotherapy-related toxicity and death was 900 pg/mL. In a multivariate Cox regression model, the hazard ratio (HR) for major chemotherapy-related toxicity and death for patients with NT-proBNP levels of >900 pg/mL were 7.3 (95% CI, 3.2-16.4; P<0.001) and 11.1 (95% CI, 3.8-32.9; P<0.001), respectively. NT-ProBNP added prognostic information independently of other well-established risk factors, including the International Prognostic Index. This marker also proved to be a predictor of death in the validation set (HR 4.4 [95% CI, 1.6-12.2] P=0.02). *Conclusion.* NT-ProBNP is a marker of major toxicity after first-line chemotherapy and death from any cause in non-Hodgkin lymphoma patients. It provides new additional prognostic information surpassing the one given by conventional lymphoma risk factors.

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CLINICAL FEATURES OF THE PATIENTS WITH LYMPHOPROLIFERATIVE DISORDERS AND ASSOCIATED AUTOIMMUNE RHEUMATIC DISEASES: SINGLE CENTER STUDY

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Background. It has been suggested that patients affected by autoimmune rheumatic diseases (ARD) particularly rheumatoid arthritis (RA),

systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS) are more prone to developing lymphoproliferative diseases (LPD). *Aims.* To analyze clinical features of LPD occurring in patients with ARD. *Methods.* 40 patients with LPD (35 non-Hodgkin lymphoma, 3 Hodgkin lymphoma and 2 myeloma) in the setting of ARD (11 RA, 15 SLE, 12 SS, 1 sclerodermia, 1 dermatomyositis) managed in the Clinic of Hematology during the period 1994-2006 were retrospectively analyzed. For the LPD cases ascertained descriptive statistics and Cox regression were calculated. *Results.* The median age of LPD patients at diagnosis was 55 years (range 33-76). There were 31 female and 9 male. In 37/40 patients ARD preceded LPD. The median interval between the onset of ARD and LPD development was 91 months (range 21-212). This interval was significantly longer in RA (113 months, range 25 - 212) than in SLE (75 months, range 21-132) and SS patients (65 months, range 29-154). In only 3 patients LPD (2 follicular NHL, 1 small lymphocytic NHL) preceded ARD (SLE 2, SS 1) with median interval time from LPD to ARD development of 39 months (range 9-72). The most frequent LPD type was NHL (35/40). The primary site of NHL was nodal in 18 and extranodal in 17 cases. The clinical stages (CS) distribution of NHL patients was as follows: CS I 5, CS II 3, CS III 13 and CS IV 14. Constitutional symptoms and bulky disease were registered in 26/35 and 3/35 cases, respectively. 32/35 NHL cases were of B-cell type (RA 8, SLE 13, SS 11) while 3 cases were of T-cell type (SLE 2, RA1). The NHL histological distribution was as follows: diffuse large B-cell lymphoma (12/35), follicular (7/35), small lymphocytic (5/35), marginal zone (5/35), Burkett NHL (2/35), anaplastic large T-cell (2/35), lymphoplasmacytic lymphoma 1/35, peripheral T-cell lymphoma (1/35). The LPD patients were treated as follows: chemotherapy alone in 22/40 (CHOP 13, COP 1, FC 2, VAD 2, ABVD 2, Chlorambucil 2), rituximab plus CHOP in 9/40 cases, chemotherapy (ABVD 1, COP 4) followed by radiotherapy in 5/40 and radiotherapy alone in 4/40 patients. The evaluation of the first line therapy showed: complete response in 16/35, unconfirmed complete response in 2/35, partial response in 8/35, stable disease in 7/14 and disease progression in 7/14 patients. NSAID, corticosteroids, cyclophosphamide, azathioprine, methotrexate and hydroxychloroquine were used for treatment of ARD before LPD development. There was no correlation between LPD histological subtype and type of antirheumatic therapy. 31 patients had died until the end of the study with a median survival of 39 months (range 3-78) after LPD diagnosis. *Summary.* Our findings are in line with mostly other reports showing more aggressive course of LPD in the setting of ARD represented in advanced clinical stages, constitutional symptoms, extranodal manifestation and aggressive histological subtype. without significant correlation between used antirheumatic drugs and LPD histological subtype.

1570

RITUXIMAB MAINTENANCE AFTER FIRST LINE IMMUNOCHEMOTHERAPY ACHIEVES EXCELLENT HIGH DISEASE CONTROL IN BOTH *DE NOVO* FOLLICULAR AND MANTLE CELL LYMPHOMAS

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Background. Rituximab (R) maintenance has demonstrated survival improvement compared with observation in patients with relapsed or refractory follicular lymphoma (FL). The efficacy and safety of RM in patients with advanced follicular lymphoma after first line immunochemotherapy is still unknown. The aim of this study was to retrospectively evaluate our experience with rituximab maintenance (RM) in *de novo* follicular (FL) and mantle cell lymphoma (MCL). *Patients and methods.* patients with *de novo* FL or MCL achieving complete remission (CR) or partial remission (PR) after first line immunochemotherapy were eligible. Three types of RM were used during study period: RM1) 375 mg/m^2/week × 4 consecutive weeks every 6 months during 2 years, RM2) R 375 mg/m^2 every 3 months during 2 years or RM3) R 375 mg/m^2 every 2 months during 2 years. Progression free survival was calculated from the day of the first R infusion and overall survival from the day of first treatment. *Results.* Forty-four patients were included: 39 (89%) FL and 5 (11%) MCL. The median age was 69 years (range 27-85) with 46% being male. Status at diagnosis: Stage I in 5%, II in 9%, III in 16% and IV in 71%; B-symptoms in 32%; IPI 0-1 in 21%, 2-3 in 58% and 4-5 in 21%. Immunochemotherapy regimens prior to RM: without anthracyclines 30%, with anthracyclines 61% and with purine analogs 9%. Status previous starting RM: CR in 35 (80%) and PR in 9 (20%). Types of RM: RM1 in 41%, RM2 in 52% and RM3 in 7%. Eight pts did not finish RM: 5 progressive disease, 1 infection, 1 delayed prolonged neutropenia and 1 exitus. Eleven pts are still receiving RM. During RM, 4 pts have progressed among CR group and 3 pts among PR group. Three out of 7 pts in the PR group have improved to CR during RM. PFS was 80% at 2 years and 74% at 4 years. PFS was significantly better for those pts receiving anthracyclin-containing immunochemotherapy prior RM compared with those who did not (91% *vs.* 61% at 2 years, P=0.025). OS was 97% at 2 years and 94% at 4 years. Toxicity was generally low, mainly minor respiratory infections. RM was prematurely withdrawn by adverse events in 2 pts (5% of the whole series): 1 pt due to recurrent infections (64 years old) and 1 pt due to delayed severe neutropenia (69 years old). Hipogammaglobulinemia was present in 42 % of cases before RM and in 39% in the last RM received. *Conclusions.* Rituximab maintenance after first-line immunochemotherapy is very active strategy for both *de novo* follicular and mantle cell lymphomas. Interestingly, anthracyclin-containing chemotherapy prior to RM has a reduced progression rate. Although, minor adverse events are seen, mainly respiratory infections, RM in first line is feasible for the majority of patients.

1571

HIGH FREQUENCY OF PRIMARY THYROID TUMORS (PTC) OCCURRING IN A GROUP OF 428 CONSECUTIVE LYMPHOMA PATIENTS OBSERVED IN A 5 YEARS PERIOD IN A SINGLE HAEMATOLOGICAL UNIT

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Background. The emergence of secondary neoplasia after treatment of a primary cancer is a more and more frequent event. The probability of a secondary cancer is due to the toxic effect of therapies and to genetic predisposition. Nonetheless epidemiological studies have highlighted that, despite previous therapies, several gene mutations and/or polymorphism are involved in the individual predisposition to multiple primary tumors. *Aims.* Evaluating the incidence of primary solid tumors in patients affected by lymphoma. *Methods.* The incidence of other primary cancers, was investigated in a series of 428 patients diagnosed with lymphoma between January 2003 and January 2010. Primary cancers were considered 1) cancer diagnosed at the same time of lymphoma in patients who had not previously received chemotherapy and/or radiotherapy 2) solid cancers that were not synchronous with the diagnosis of lymphoma but diagnosed in patients who were not treated with chemotherapy and/or radiotherapy, with the exception of those patients who received radiotherapy outside the field of tumor occurrence. Patients suffering from Sjogren syndrome were excluded from the analysis. *Results.* 21 patients (4.9%) out of 428 consecutive lymphoma patients, had a diagnoses of a primary solid tumour and overall 25 cancers were diagnosed. The relative frequency (RR) of the different tumours was the following: thyroid 32% (8 cases), renal 16% (4 cases), melanoma 12% (3 cases), breast 15% (2 cases), lung 8% (2 cases), uterus 15% (2 cases), liver 4% (1 case), colon 4% (1 case), myxofibrosarcoma 4% (1 case), tongue 4% (1 case). *Conclusions.* The RR of PTC (32%) in our group of lymphoma patients appears to be unexpectedly high compared to the RR of PTC in the Italian Register of Tumours (1.6%), suggesting possible common mechanism or similar pathogenetic pathways. This association deserves further studies to be elucidated.

1572

REVISED INTERNATIONAL PROGNOSTIC INDEX (R-IPI): A NEW PROGNOSTIC MODEL FOR DIFFUSE LARGE B-CELL LYMPHOMA PATIENTS

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Background. In the last decade, anti-CD20 monoclonal antibodies have modified treatment strategies for diffuse large B-cell lymphoma (DLBCL), leading to a marked improvement in patients outcome. The International Prognostic Index (IPI) is the prognostic score most commonly used to predict overall survival (OS) and progression free survival (PFS) in patients with DLBCL. However, it was designed before the addition of Rituximab (R) to the standard chemotherapy regimens. Recently, a new modified IPI score (R-IPI) has been described for Rituximab-treated patients. *Aim.* To compare the standard IPI and R-IPI model scores

in patients with DLBCL treated with R-containing regimens. *Methods.* A retrospective analysis of 100 patients (median age [range] 62 years [19-89]) diagnosed with CD20 positive DLBCL was performed in Hospital del Mar, Barcelona. First line treatment consisted of R monotherapy (n= 4), R-CHOP (n= 59), R-CMyOP (n= 20), R-EPOCH (n= 8) and R-another chemotherapy schedules (n=9). *Results.* Distribution of patients by standard IPI and R-IPI, and OS and PFS at 48 months according to the IPI and R-IPI were as follows (Table 1). Median follow up was 35 (range 1-87) months. OS and PFS of the whole cohort were 70% and 67.5% at 4 years, respectively. Median OS and PFS were 61 months (CI 95%, 53-69%) and 62 months (CI 95%, 54--70), respectively. *Conclusions.* 1. Standard IPI score and R-IPI model can identify different risk groups of patients with statistically significant different OS and PFS. 2. R-IPI model is slightly better for stratifying low risk DLBCL patients in the Rituximab® era.

Table 1.

IPI Risk Groups	patient n	OS (%)	PFS (%)	R-IPI Risk Groups	patient n	OS (%)	PFS (%)
Low 0-1	42	87	85	Low 0	14	100	91
Low-Intermediate 2	16	71	80	Intermediate 1-2	44	78	75
Intermediate-High 3	15	68	55	High 3-4-5	42	51	49
High 4-5	27	43	34				
p		0.02	0.00	p		0.01	0.02

1573

TREATMENT OF SPLENIC MARGINAL ZONE LYMPHOMA

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Splenic marginal zone lymphoma (SMZL) is well known lymphoma. *Aim.* Our goal is to characterized SMZL and shows the most effective method of treatment. *Material and methods.* 86 patients (33 males, 53 females) with average age 59,6 years were included in the study. The diagnosis based on a typical histological picture and immunophenotype. We gathered demographic and clinical data, blood counts at presentation, spleen and liver sizes, lymphadenopathy, the presence of monoclonal component, anti-HCV, anti-HBsAg antibodies, lesions of bone marrow, cytogenetic findings, treatment, type of response and duration. Follow up was from 3 months to 7 years. Splenomegaly and bone marrow involvement was diagnosed in all cases, hepatomegaly in 60,5% cases, visceral lymphadenopathy, high level of LDH and B-symptoms in half of cases, combination with AIHA in 6 cases, positive serology for viral hepatitis in 13 patients. In 5 cases we are identified aberrant phenotype with $CD5^+$ expression. In half of patients we revealed cytogenetic abnormalities (trisomy 3, 12, 18, deletion 7, t(14;19)(q32;q13)). Depending on the initial treatment strategy, all patients were divided into groups: I group - untreated patients, II group - patients who have chemotherapy held as first-line therapy, III group - patients who performed a splenectomy, IV group - patients who performed a splenectomy and subsequent chemotherapy. *Results.* In II group after an average of 6 months had a progression and progression-free survival (PFS) was 0%. In the III group, PFS (in terms of monitoring up to 7 years) was 70% (P=0.01). Overall survival III group was about 95% (for a period of observation up to 7 years). *Conclusion.* SMZL is a heterogeneous, due to the clinical course (secretion of paraprotein, the combination with AIHA), different cytogenetic abnormalities, aberrant expression of CD5. Splenectomy is the first-line therapy in patients with SMZL, which confirmed the data PFS, which amounted to the deadline of 7 years was 70% (P=0,01).

1574

PRIMARY NODAL DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL), STAGE I-II. CLINICOBIOLOGICAL FEATURES AND PROGNOSTIC FACTORS: EXPERIENCE IN A SINGLE INSTITUTION

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Background. DLBCL is an aggressive and potentially curable lymphoma. It presents itself as a localized disease in 30% of all cases and as primary nodal in 50-60% of these. *Aims.* To analyze the main clinicobiological characteristics, response to therapy, outcome and prognostic factors. *Methods.* Ninety four patients (42/52 M/F; median age, 60.5 years) with a novo primary nodal DLBCL, Ann-Arbor I-II stage were diagnosed in our centre during a 17-year period and recorded in a data base. *Results.* Twenty-eight patients (30%) were stage I and 66 (70%) stage II. The distribution according to IPI were as follows: low risk, 44 (47%); low-intermediate risk, 37 (39.4%) and high-intermediate risk, 13 (13.6 %). The main clinicobiological features are in the Table. The treatment consists of: fifty one patients were treated with CHOP-like chemotherapy, forty two with CHOP-like plus rituximab chemotherapy and radiotherapy (1). Complementary radiotherapy (C-RT) was administered in 61%. C-RT was associated with a significant overall survival (OS) and event free survival (EFS) at 5 years (77% *vs.* 58%; P=0.015 and 76% *vs.* 52%; P=0.019 for patients who received C-RT respect the ones that didn´t receive C-RT). Response rates were as follows: 82% complete response (CR), 9.5% partial response and 8.5% failure. The overall survival (OS) at 5 years was 70% (CI 95%: 60-80%). CR and OS were slightly higher in regimens containing rituximab than without rituximab (83% *vs.* 80%, respectively; P=0.31, y 73% *vs.* 68%, respectively; P=0.43). The median follow-up was 4.2 years. 16 of 77 patients in CR eventually progressed, with a event free survival at 5 years 66% (CI 95%: 56-76%). Thirty-two patients died (progression, 28; sepsis, 2; acute ischemic stroke, 1 and gastric adenocarcinoma, 1). The IPI at diagnosis and β2-microglobulin were the main prognostic factors (OS at 5 years: 84%, 60% and 47% for patients with low, low-intermediate and high-intermediate risk, respectively; P=0,001; OS at 5 years: 80% and 58% for patients with high and low β2-microglobulin, respectively; P=0,014). *Summary.* localized nodal DLBCL have a particular clinicobiological features. C-RT seems to be associated with better OS and EFS. IPI and β2-microglobulin are the main prognostic factors. The exact rule of treatment with rituximab need to be investigated.

Table.

	DLBCL (N=81)
ECOG 0-1 (%)	73
B symptoms (%)	16
Bulky disease (%)	40
LDH > 3.4 ukat/L (%)	87
β2-microglobulin > 2.2 mg/L (%)	47
IPI low/low-intermediate risk (%)	86
Bcl2+ (%)	87
Bcl6+ (%)	50
CD10+ (%)	30
CR (%)	82
OS 5y (%)	70

1575

DRAMATIC RESPONSE TO LENALIDOMIDE OF A REFRACTORY CUTANEOUS DIFFUSE LARGE B-CELL LYMPHOMA,LEG-TYPE

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Background. Primary cutaneous diffuse large B-cell lymphoma, leg-type (PCLBCL-LT) is a distinct entity which mostly occurs in the elderly and which has a predilection for the legs. It is characterized by confluent sheets of large cells, with a round morphology and a strong expression of BCL2, Foxp3 and IRF4, without IRF4 rearrangement. As opposed to other primary cutaneous B-cell lymphoma, PCLCCL-LT

has a poor prognosis with a five-year survival rate around 50%. Moreover, while initial responses are usually obtained with a combination of chemotherapy and rituximab, skin but also extra-cutaneous relapses occur in 50% of patients. We report herein a dramatic response to lenalidomide in a PCLBCL-LT refractory to various treatment protocols. *Case.* A 75 year old woman presented with a dark purple nodule on the left leg in September 2005. Skin biopsy showed dense proliferation of centroblasts with a CD20, BCL2, BCL6, MUM1 positive staining and high proliferative index as all the cells expressed Mib1. A B-cell clone was detected by PCR. PET scan detected multiple foci on the same leg and a spread to the inguinal lymph node (SUV >4). An initial complete response was obtained by May 2006 after 6 cycles of R-CHOP associated with radiotherapy. From November 2007, skin recurrences were treated successively with radiotherapy, ibritumab tiuxetan and then six cycles of Mini-CHVP + rituximab, followed by rituximab maintenance every two months. In April 2009, a fourth skin recurrence, associated with neurogenic pain along the path of the sciatic nerve, led to a regimen of Holoxan-VP 16. This treatment was ineffective with progression of tumors on the same leg (Figure 1A). Skin biopsy showed a similar histology and phenotype profile. FISH analysis did not detect IRF4 gene rearrangement and a B-cell clone identical to the initial one was found by PCR. Finally, in September 2009, the patient was prescribed single-agent lenalidomide (10 mg/day), administered in cycles of three weeks separated by one-week wash-out periods. Within one month, all lesions had regressed (Figure 1B) and the neurogenic pain had disappeared. Six months later, the patient was maintained on lenalidomide (20 mg/day) with no recurrence and no clinical or biological adverse reactions. *Conclusion.* The encouraging rapid complete response observed in this patient suggests that lenalidomide may be safe and effective in relapsed or refractory aggressive lymphoma such as PCLBCL-LT. To our knowledge, this is the first time that such a case has been reported. Carefully designed studies are needed to determine the place of lenalidomide in PCLBCL-LT management as well as its biological effects possibly through a down regulation of the IRF4 and NF-KB pathway that has been demonstrated to be involved in PCLBCL-T.

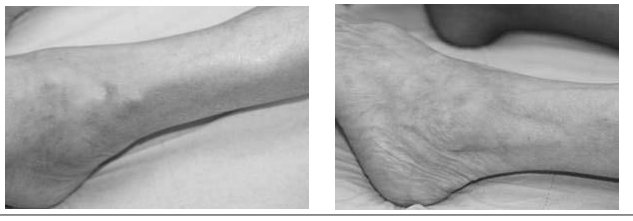

Figure. Left leg in September 2009 (1A) and after one month of lenalidomide treatment (1B).

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RITUXIMAB PLUS CVP (CYCLOPHOSPHAMIDE, VINCRISTINE, AND PREDNISOLONE) COMBINATION CHEMOTHERAPY IN ADVANCED STAGE MARGINAL ZONE B-CELL LYMPHOMA AS A FIRST-LINE THERAPY

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Background and Aims. Although successful results were reported with local treatment or several antibiotics for localized marginal zone B-cell lymphoma (MZBCL), it presents as a disseminated disease in one-third of the cases at diagnosis and relapses involving distant sites after local therapy have been reported. We performed multi-center, phase II trial to investigate the efficacy and safety of rituximab plus CVP (R-CVP) combination chemotherapy in patients with previously untreated stage III/IV MZBCL. Preliminary results of this trial previously reported at the 14th EHA (abstr 980), in here, we report updated results. *Methods.* Patients received rituximab 375mg/m^2 on day1 of each cycle. CVP consisted of cyclophosphamide 750 mg/m^2 and vincristine 1.4 mg/m^2 (maximum 2.0 mg), given intravenously on day 1, and oral prednisolone 100 mg on days 1-5. The treatment was repeated every 3 weeks and continued for 6 or 8 cycles until disease progression, withdrawal due to toxicity, or withdrawal of consent. *Results.* Between March 2006 and July 2008, a total of 42 patients were enrolled with informed consent at this trial from 13 institutes in Korea. Among these patients, 2 patients were dropped out after 1 and 2 cycles of chemotherapy without evaluation. The median age of the evaluated 40 (24 males, 16 females) patients is 56 (range, 29-77) years. Thirty three patients (82.5%) had extranodal sites involvement, 19 (47.5%) of who had 2 or more sites involved. The IPI score were 1 in 10 (25%), 2 in 17 (43%), 3 in 11 (27%), and 4 in 2 (5%) patients. The patients received a total of 287 cycles of R-CVP chemotherapy (range 3 - 8 [median 8] cycles/person). There were 24 CR (60%), 11 PR (27.5%), 4 SD (10%), and 1 PD (2.5%), making response rate 87.5% (95% confidence interval, 77.1 - 97.9%). There were 30/287 cycles (10.5%) and 5/287 cycles (1.7%) of grade 3/4 neutropenia and febrile neutropenia, respectively. Non-hematologic toxicities were mild and tolerable. There were 19 cycles (6.6%) of delayed chemotherapy (median 1 week) mainly because of neutropenia (10 cycles) and non-hematologic toxicities (6 cycles). Dose reduction was needed in 9 patients (23 cycles) for cyclophosphamide and in 1 patient (4 cycles) for vincristine. There was toxicity-related hospitalization in 9 patients (22.5%) during treatment. But, there was no treatment-related death. After a median follow-up of 30.7 months (range, 11.1-43.8 months), the estimated three-year progression-free survival and overall survival were 59.5% and 95.0%, respectively. *Conclusions.* R-CVP regimen seems rather effective and tolerable in the treatment of advanced stage MZBCL. Longer follow-up period for these patients and other comparative studies are warranted to verify the results of this trial.

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PROPOSAL OF NEW DISEASE EXTENT GROUPS IN PATIENTS WITH PRIMARY EXTRANODAL DIFFUSE LARGE B CELL LYMPHOMA

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Background. The incidence of primary extranodal non-Hodgkin's lymphoma (NHL) is increasing worldwide. The primary extranodal NHL is very heterogeneous disease and there is a variety of opinions about the uniform application of the traditional Ann Arbor staging system to primary extranodal NHL. *Aims.*We explored modification of disease extent groups for patients with primary extranodal diffuse large B cell lymphoma (DLBCL). *Methods.* We retrospectively evaluated 249 patients with primary extranodal DLBCL who diagnosed between July 1993 and July 2007. We applied various disease extent grouping systems and compared to Ann Arbor staging system. *Results.* The median age was 55 years (range, 15-84). The gender distribution was 49.0% (122/249) men and 51.0% women (127/249). The stomach was the most common primary extranodal site (30.1%, 75/249). Ann Arbor staging system could not discriminate appropriately the survival among stage 1 through 4. Disease extent groups in figure showed the best discrimination. According to modified disease extent groups, especially group 4 showed significantly worse outcome compared to other groups (P<0.05). *Summary/Conclusions.* Ann-Arbor system cannot be easily applied to primary extranodal DLBCL. Modified disease extent grouping system of current study could be useful for patients with primary extranodal DLBCL.

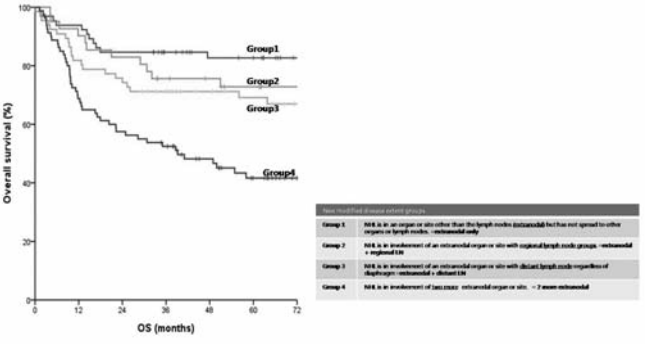

Figure. Modified disease extent groups.

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EXPRESSION OF C-FLIP IN NODAL DIFFUSE LARGE B CELL LYMPHOMA (DLBCL): AN IMMUNOHISTOCHEMICAL STUDY

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Background. C-FLIP is one of the important antiapoptotic proteins that have been shown to be overexpressed in various tumor cells. It was shown that downregulation of c-flip results in sensiting resistent lymphoma cells, but data about his clinical and prognostic significance in patients with DLBCL are not consistent. The aim of this study was to evaluate the incidence and prognostic significance of c-FLIP expression in patients with nodal DLBCL, as well as the relationship of their expression with selected clinical data, histological features and proliferative activity of lymphoma cells. *Methods.* We studied 54 patients with primary nodal DLBCL. Immunohistochemistry was performed on formalin/fixed, paraffin/embedded tissue section of lymph nodes with monoclonal antibody against c-FLIP. Expression of c-FLIP was analyzed using standard immunohistochemical method on routinely processed paraffin-embedded lymph node specimens. Cut off value of 40% was used for discriminate c-FLIP positive and c-FLIP negative sections. The patients were treated with CHOP or R-CHOP chemotherapy regimen. *Results.* The group consisted 30 of men and 24 women with median age of 54,98 years. Advanced stage (III/IV) was observed in 37 (68.52%) cases, and distribution according to the IPI was as follows: low risk 16 (29.62%) patients, low intermediate risk 21 (38.88%), high/intermediate risk 11 (20,37%) and high risk 6 (11.11%) patients. Overall response rate was achieved in 42 patients (64.45%). A complete response (CR) was achieved in 31 patients (73.8%). C-FLIP expression was detected as diffuse cytoplasmatic staining in 23 (42.59%). There was no significant correlation between c-FLIP expression and age, gender, clinical stage, IPI, hemoglobin concentration, platelet count and histological type. No association was observed between the expression of c-FLIP and proliferative activity index (measured with Ki-67 immunoreactivity), subtype (GCB/ABC) and response to therapy. No significant difference was observed regarding overall survival between c-FLIP positive and c-FLIP negative patients (P=0.410). *Conclusion.* Our results have shown that c-FLIP has not the prognostic significance in patients with nodal DLBCL. However, larger studies are mandatory to confirm these observations.

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MULTIPLE MUCOSA-ASSOCIATED LYMPHOID TISSUES (MALT)-ORGAN INVOLVED MARGINAL ZONE B-CELL LYMPHOMA: CONSORTIUM FOR IMPROVING SURVIVAL OF LYMPHOMA (CISL) STUDY

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Background. According to previous review, multiple mucosa-associated lymphoid tissues (MALT)-organ involved marginal zone B-cell lymphomas (MZLs) are present in 10%~30% of patients. But, specific relationship among involved organs, clinical features, and prognosis were not clearly identified. *Aims.* In this study we conducted retrospective analyses of multiple MALT organs involved MZLS (MM-MZLs) to identify their clinical features, treatment, and prognosis. *Methods.* For analysis, between Jun. 1987 and Jun. 2009, a total of 55 patients with histologic diagnosis of MM-MZL from 17 different institutions in Korea were included. MM-MZL was defined that MZL involved more than 2 different MALT organs. Multiple involvements within one MALT organ (ex- multiple lung nodules, multiple stomach lesions) were excluded. *Results.* The male/female ratio of the 55 patients was 41 to 14. The median age of our subjects was 59 years (range: 30-82 years). MM-MZL without lymph node (LN) or was only 9 patients (36.2%). BM involved observed 17 patients (30.9%). The most common site of involvement was gastrointestinal (GI) tract (25 patients-45.5%) followed by lung (40%), waldeyer's ring (WR) (27.3%), and ocular area (25.5%). Ocular MZLs were commonly accompanied with WR- or lung-MZLs. GI- MZLs were WR or GI-MZLs. Lung MZLs were frequently observed with ocular and GI-MZLs. WR-MZLs were ocular or GI-MZLs (Figure). 53 patients had treated -2 watchful wait. 48 patients had received chemotherapy based treatment. Out of them, CR or PR was achieved in 38 patients (79.2%, 95% CI, 67-91%). Median time to progression (TTP) was 2.3 years (95% CI, 1.4-3.2 years). Cause-specific overall survival (OS) was not reached median value. 5 year OS rate was 84.9%. There were no differences between MM-MZL alone and LN or BM involved MM-MZL in median TTP (5.7 years *vs.* 2.0 years, P=0.271) *Conclusion.* MM-MZLs tend to be an indolent disease - characterized by prolonged survival with frequent relapses. The majority of them was controlled well with chemotherapy based treatment, and could achieve prolonged survival. GI, lung, WR, and ocular area were commonly presented with other MALT site MZL and it seems to be existing organ relationship within MM-MZLs.

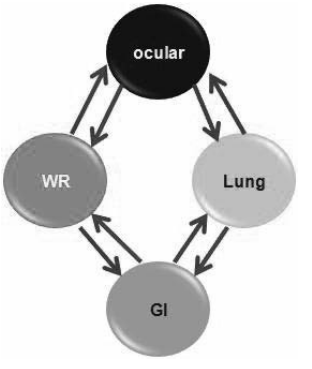

Figure. Organ relation.

1580

MODIFIED HYPERCVAD AS AN EFFECTIVE TREATMENT FOR PLASMABLASTIC LYMPHOMA

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Background. Plasmablastic lymphoma (PBL) a distinct subtype of NHL, is classified as an individual entity by the WHO based upon morphological, immunological and clinical features.[1] It typically occurs in the setting of HIV infection, with a predilection for the oral cavity. It consists of lymphocytes of immunoblastic morphology, absent or weak expression of CD45 & CD20, but expression of plasma cell related antigens like CD138. Ki-67 index is generally >90% and about 60% of cases are said to be EBV-related. Since its first description in 1997 by Delecluse[2] there have been more than 100 cases reported. Over time, the disease's clinical spectrum has expanded, with a number of case reports in HIV-negative and extra-oral sites as well. Inspite of this, there still is no standard treatment and it is continues to be associated with poor prognosis with a high relapse rate. *Aims.* We describe 4 patients with PBL successfully treated with a modified HyperCVAD at a single centre. *Methods.* HIV status was known in 2 of the 4 patients and both were already on HAART. In the other 2 patients, HIV was diagnosed after tissue biopsy had confirmed the nature of the lymphoma. Patient characteristics are summarised in Table 1. Disease staging included CT scan of thorax, abdomen and pelvis and bone marrow biopsy. Following completion of treatment response was confirmed by CT scan, supplemented by a PET scan. All 4 patients were treated with the 3 cycles of modified HyperCVAD alternating with methotrexate/cytarabine (Table 2). The 4th patient also had localized radiotherapy at the end of the chemotherapy. Intrathecal chemotherapy was administered with prophylaxis intent as there was no evidence of CSF infiltration. All patients were also on concomi-

tant HAART and prophylaxis with fluconazole, acyclovir and dapsone. There were no major complications throughout the treatment course. *Results.* All the 4 patients achieved CR and continue to be in remission shortest being 10 months and the longest 4 years. *Discussion.* Castillo3 presented a comprehensive summary of 112 published cases, concluding CHOP failed to show an association with survival. This is not surprising, as this is an aggressive lymphoma with high proliferation index. A good response to HAART was usually associated with better prognosis. Unlike CHOP, CODOX-M/IVAC, a chemotherapy regime most commonly used with aggressive lymphomas has been used with more success.[3,4] However it is an inpatient regime, more toxic than HyperC-VAD and treatment related deaths have been reported.4 CD20 negativity means that Rituximab does not have any role in its management. Modified Hyper CVAD regime we used with slight alterations from the original regime described for acute lymphoblastic lymphoma. Cyclophosphamide was given 12 hours apart for the 1st 2 doses, and then as a single daily dose, thus achieving outpatient treatment. Day 11 to 14 dose of dexamethasone as originally described was omitted. *Conclusion.* These 4 cases show HyperCVAD to be an effective alternative to CODOX-M/IVAC option for this rare lymphoma, with good safety profile. *(Note Table 1, 2 & references not included).*

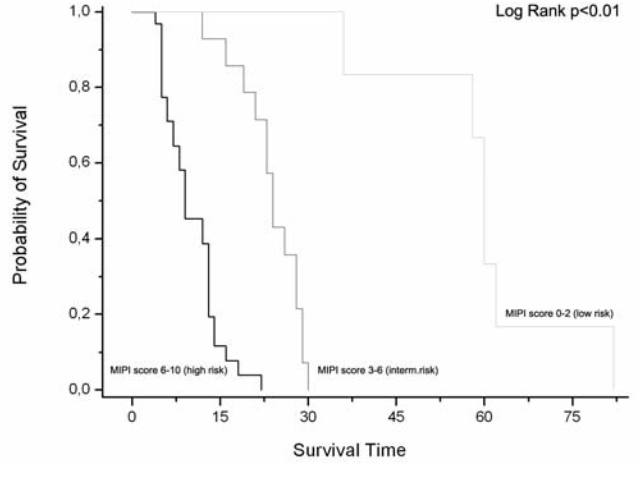

Figure. Abdominal mass in patient 3.

1581

INTERNATIONAL PROGNOSTIC INDEX (IPI) VS. MANTLE-IPI (MIPI) RELATION IN THE PATIENTS WITH ADVANCED STAGE MANTLE CELL LYMPHOMA (MCL): THE CORRELATION BETWEEN LABORATORY DATA AND CLINICAL OUTCOME

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Background. Mantle cell lymphoma (MCL) is a hematologic malignancy with affected B-cell lineage on the basis of specific molecular genetics involving cyclin D1 gene over-expression and resulting pathological alteration. Extranodal involvement and low-level answer to therapy are typical for MCL patients. Most of them have advanced stage of disease. *Aims.* to evaluate international prognostic index (IPI) *vs.* mantle-IPI (MIPI) in correlation with immunophenotype and clinical characteristics in advanced stage MCL patients and their influence on overall survival (OS). *Methods.* Mantle-international prognostic index (MIPI) was scoring by each patient according to standard recomendations including: age, leukocyte count, LDH value and ECOG performance status. In low risk group are patients with MIPI 0-3, intermediate risk 4 and 5 and high 6-10. Bone marrow (BM) and/or peripheral blood (PB) derived cell flow cytometric analyses for following antigens (Ag) were performed: HLA-DR, CD19, CD20, CD22, CD23, CD25, CD10, SmIg, kappa, lambda, CD79b, CD38, FMC7, CD3, CD2, and CD5. Cut-off for Ag expression was accepted positivity > 30%. Pathohistology and immunohistochemical testings were performed in BM an lymph node biopsies. The used monoclonal antibody specifies were: CD5, CD20, CD23, CD10, CD79b, and cyclin D1. The influence between immunophenotype and clinical parameters was estimated by Spearman's correlation. The survival curves were estimated using the Kaplan-Meier method. The log rank test evaluated association between overall survival (OS) and clinical characteristics, IPI and MIPI. The use of the Cox proportional hazards model determinated that independent prognostic factors influenced OS. *Results.* A total of 54 patients diagnosed between 1996 and 2009 were evaluated. There was 17 patients in IV CS, while 37 patients had leukemic phase at presentation. Among patients, 46 were treated with CHOP, 2 with FND, and 6 with Hyper-CVAD as initial treatment option. Typical immunophenotype was presented in all cases: $CD5^+$, $CD23^-$, Cyclin $D1^+$. Pathohystological type of BM infiltration was diffuse (63% of patients) and in remainder nodular. Hematological parameters showed median Hb 113g/L, Plt 121×10^9/Ll, WBC 29×10^9/L. Extranodal involvement (22%) included pleural effusions, bowel, sinus and palpebral infiltration. Among patients, 9 of them achieved complete and 23 partial remission. Median OS was 23 months, and there were no significant OS-differences between CS IV and leukemic phase patients. Survival analyses showed that negative prognostic influence had high IPI (P<0.001), MIPI (P<0.001), presence of extranodal localization (P<0.01), and diffuse type of BM involvement (P<0.01). The level of IPI correlated more with the age of patient with high statistic significance (P<0.001). Regarding the immunophenotype of malignant cells, higher expression of CD5 and CD22 correlated with high MIPI value and short survival, with low statistic significance (P<0.05). Using Cox regression according to OS, MIPI had independent prognostic value (P<0.001). *Conclusion.* In the advanced MCL, higher MIPI, IPI, diffuse BM infiltration, extranodal disease localization had negative influence on survival. In contrary to IPI, MIPI value reflects much more biological characteristics of disease.

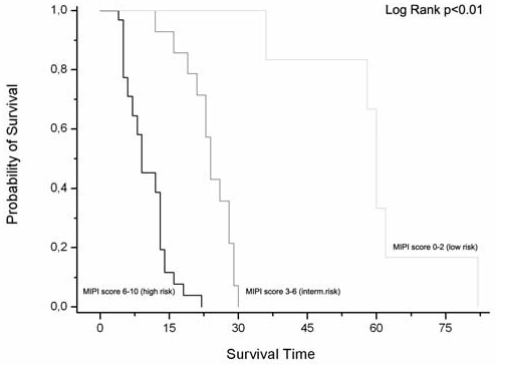

Figure. Survival according to different MIPI values.

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ADDITION OF RITUXIMAB TO HIGH-DOSE METHOTREXATE-BASED CHEMOTHERAPY IMPROVES OUTCOMES IN ADULT BURKITT LYMPHOMA PATIENTS

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Background. Burkitt lymphoma (BL) is a very aggressive B-cell lymphoid neoplasm. Results of treatment have been substantially improved by the introduction of high-dose methotrexate-based chemotherapy with overall survival rates in adult patients with disseminated disease ranging from 40-60%. BL tumor cells express CD20, but there is a dearth of data on the effect of anti-CD20 therapy in this disease. *Aims.* To analyze the influence of the addition of rituximab to a standard chemotherapy regimen in adult patients with sporadic BL. *Methods.* This is a retrospective study performed by chart-review of all adult patients with sporadic BL treated at our center with the modified B-NHL 86 regimen adopted from the German Multicenter Study Group for the Treatment of Adult ALL (GMALL) consisting of high-dose methotrexate, ifosfamide, vincristine, cytarabine, cyclophosphamide, doxorubicine and etoposide with central nervous system prophylaxis. Six cycles of treatment were planned. Since 2006 all newly diagnosed patients also received rituximab, 375 mg/m^2 per cycle. Patients in PR after the end of treatment received involved-field radiotherapy. We identified 19 patients, 15 men and 4 women, 16-61 years old (median 35) with BL stage II-IV. Eight were treated without and 11 with rituximab. *Results.* Toxicity of the treatment was substantial. Three patients, 1 in the rituximab and 2 in the chemotherapy-only group, died early after diagnosis due to multiple organ failure. All surviving patients had

episodes of grade III or IV febrile neutropenia, 1 in the rituximab and 1 in the chemotherapy-only group died. Of the remaining 5 patients in the chemotherapy-only group, 2 relapsed during treatment and died shortly thereafter. The remaining 3 patients all achieved CR. None of the patients in the rituximab-treated group was refractory to treatment or relapsed. At the end of treatment 6 had CR and 3 PR. The difference in response to treatment is statistically significant (38% *vs.* 82 %, P= 0.048, Pearson chi-square test). None of the patients in remission after the end of treatment relapsed. With a median follow-up of 47 months, progression-free and overall survival in the chemotherapy-only group is 38% and in the rituximab-treated group 82% (P=0.060, log-rank test) (Figure 1). *Conclusions.* The addition of rituximab to a high-dose methotrexate-based chemotherapy regimen improves response rates, progression-free and overall survival of adult patients with disseminated sporadic BL. The toxicity of this type of treatment is substantial; mortality is most frequently caused by metabolic complications at the beginning of the treatment.

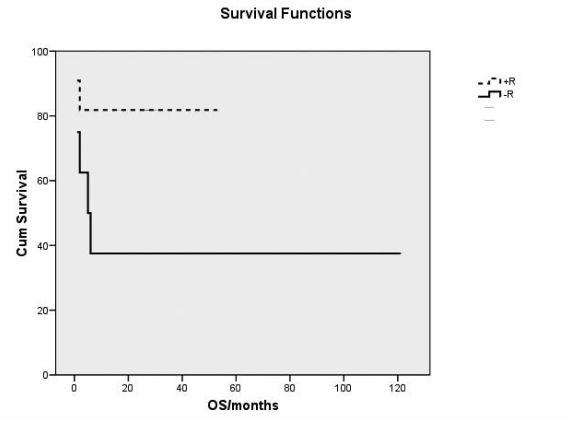

Figure 1.

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PROGNOSTIC VALUE OF FDG-PET BEFORE AUTOLOGOUS TRANSPLANTATION IN RELAPSING/REFRACTORY HODGKIN'S LYMPHOMA: A SINGLE CENTRE EXPERIENCE

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Background. FDG-PET (PET) has assumed a relevant role in the management of patients with Hodgkin's Lymphoma (HL) in recent years. PET performed after two courses of ABVD chemotherapy is presently considered the most relevant available prognostic factor and correlates with survival. Recent data show a high prognostic value of pre-transplant PET in patients with recurrent or refractory HL. *Aim.* to evaluate the role of PET in predicting outcome before high dose chemotherapy and autologous stem cell transplantation (ASCT) in resistant or relapsed HL. *Methods.* We retrospectively evaluated 20 patients (pts) (8M, 12F, median age 39 years, range 16-52) treated with 3 courses of IGEV followed by peripheral blood stem cell collection and BEAM conditioned ASCT, from April 2005 to February 2008. At the time of enrolment, B symptoms were present in 8 pts (40%), bulky disease in 2 pts (10%), extranodal involvement in 4 pts (20%) and the International Prognostic Score was >2 in 6 pts (30%). After first line chemotherapy, 11 pts were resistant and 9 pts were in first relapse. PET was performed at relapse/progression and after the third cycle of IGEV, before ASCT. Univariate analysis was performed to assess the correlation of progression free survival (PFS) with the pre-transplant PET result, presence of bulky disease, extranodal disease and chemoresistance/chemosensitivity to pre-transplant therapy evaluated by CT scan. *Results.* The pre-transplant PET evaluation was positive in 14 pts (70%) and negative in 6 (30%). Among pts with a positive pre-transplant PET evaluation, 8 (57%) progressed after a median follow-up of 13 months, 5 pts (35%) died of lymphoma-related death, 5 pts (35%) relapsed and 1 pt (7 %) is in continuous complete remission at 9 months. Among the 6 pts with a negative pre-transplant-PET evaluation, 5 pts (83%) are in continuous complete remission after a median follow-up of 14 months and 1 pt (16%) relapsed after a follow-up of 20 months. After a median follow-up of 15 months, among the parameters analyzed only pre-transplantation PET significantly influenced the PFS (P=0,01). *Conclusion.* This retrospective study shows that the pre-transplant PET result is significantly correlated to outcome after ASCT in patients with recurrent or refractory HL. Multicentric studies in larger patients cohorts are warranted to confirm these preliminary data.

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A UNIQUE PATTERN OF EXTRANODAL INVOLVEMENT IN KOREAN ADULT SPORADIC BURKITT LYMPHOMA

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Background. Extranodal involvement is very common in patients with Burkitt lymphoma (BL). The pattern of extranodal involvement might have regional diversity with prognostic implications. We evaluated the pattern of extranodal involvement and their impact on clinical outcomes in Korean adult patients with BL. *Methods.* Total 64 patient data of Burkitt lymphoma was available between 1996 - 2009 in Asan Medical Center Non-Hodgkin's Lymphoma registry (n=1,725). We performed retrospective analysis of patient data and characterized the distribution of extranodal involvement sites of BL. *Results.* Median age was 48, ranging from 16 to 81, and male: female ratio was 1.28:1. Only seven patients (10.9%) had B symptoms. Median LDH level was 455.5 U/L and 45 patients (70.3%) had elevated LDH level (>250 U/L). Thirteen patients (20.3%) were with ECOG (Eastern cooperative oncology group) performance status ≥2. Ann Arbor stage IV was predominant (40 patients, 62.6%). Most of the patients had extranodal involvements of BL (57/64, 89.0%). Thirty-seven patients (57.8%) had low or low-intermediate risk and 27 patients (42.2%) had high-intermediate or high international prognostic index (IPI). Thirty-four patients (53.1%) had two or more extranodal involvement sites. Stomach (26.6%) was the most common extranodal involvement site. Small and large intestine (25%), bone marrow (23.4%), genitourinary tract (21.9%), and bone (18.8%) were also common extranodal involvement sites. Stomach, genitourinary tract and bone are unique extranodal sites relatively more frequently involved compared to previous reports. Involvement of central nervous system, which was common in western reports, was in 2 patients (3.1%). Patients with involvement of small and or large intestine showed better complete response rate than those who were not involved (100% *vs.* 62.1%, P=0.037). Two-year overall survival was significantly lower in patients with bone marrow involvement (33.3% *vs.* 73.3%, P=0.027), central nervous system involvement (0.0% *vs.* 67.6%, P=0.025). Patients with two or more extranodal involvements showed worse overall survival rate (47.6% *vs.* 83.3%, P=0.041). *Conclusions.* Stomach, genitourinary tract, and bone were uniquely more frequent extranodal involvement sites in Korean adult patients with BL compared to previous reports. Central nervous system involvement was not common. Bone marrow and central nervous system involvements were associated with poor survival.

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HIV-ASSOCIATED NON-HODGKIN'S LYMPHOMA: A SINGLE CENTRE RETROSPECTIVE STUDY

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Background. The increased risk for B-cell non-Hodgkin's lymphoma (NHL) development in patients with human immunodeficiency virus infection (HIV) is confirmed in many studies published so far. But, in the absence of randomized trials, the prognostic factors and the optimal treatment of the patients with HIV-associated NHL remain a matter of debate. *Aim.* The aim of our study was to analyze clinical features, prognostic factors, optimal treatment and outcome in patients with HIV-associated NHL. *Patients and methods.* Our study included 34 pts with HIV- associated NHL treated at the Clinical Center of Serbia during the period September 1996 to January 2010. The median patient age was 49±12,7 (range 32-83); 23/11 (68%) were males; 16 pts (47%) had a previous AIDS diagnosis; 32 pts (94%) had an aggressive type of lymphoma (DLBCL, Burkitt); EN presentation had 23 pts (68%). Accord-

ing to the IPI score, 5/20 pts had an IPI=0-2 and 15/20 had an IPI=2-4. Chemotherapy (ldmBACOD, CHOP, EPOCH) received 26 pts (76%), while 19 (56%) were treated with combination chemotherapy (CT) and highly active antiretroviral therapy (HAART). Number of $CD4^+$ cells was quantified by flow cytometry. Analyses included the comparison of aforementioned parameters in two subgroups: survivors and those who died. The median follow up was 26±32 (range 1-120) months. *Results.* The median IPI score for survivors was 1,4±1,1 while pts who died had IPI score 2,8± 0,9. Patients with IPI score <2 had a significantly better survival than pts with IPI score ≥2 (log rank P=0,018). Six pts (18%) achieved complete or partial remission with median survival 60±24 months while 28 (82%) were non-responders with significantly shorter median survival 19±29 months (log rank P<0,01). The median survival of pts treated with combination CT+HAART was 60 months while in a subgroup treated with CT alone was less than 12 months (log rank P<0,01). The median CD4+ cell count among survivors was higher but without significance for survival (246±91/μL *vs.* 131±36/μL P=0,058). *Conclusions.* The present data showed that the most reliable prognostic factor for the overall survival in patients with HIV-associated NHL is an IPI score and a type of applied therapy (HAART and CT). Studies with larger numbers of patients and randomized trials are needed in order to confirm our data.

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CLINICOPATHOLOGIC CHARACTERISTICS OF A CASE SERIES OF PRIMARY SOFT TISSUE DIFFUSE LARGE B-CELL LYMPHOMA (ST-DLBCL). COMPARISON OF OUTCOME BETWEEN ST AND NONE ST-DLBCL

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Background. Extra nodal involvement is common in Diffuse Large B-Cell lymphoma (DLBCL). Primary soft tissue DLBCL (ST-DLBCL) is a rare clinical presentation that can mimic sarcoma. Only few studies have described the characteristics of soft tissue non-Hodgkin lymphoma (ST-NHL) and none have focused on DLBCL. *Aim.* This retrospective study analyzed the clinicobiological features at diagnosis, response to therapy of ST-DLBCL and compared their outcomes with none ST-DLBCL. *Methods.* All patients with primary ST-NHL diagnosed between January 1981 and December 2007 and followed up in our institution were selected. We identified one Burkitt lymphoma, one ALK-negative anaplastic large cell lymphoma, one secondary transformation of a known Waldenstrom lymphoma and eleven DLBCL. Among these 11 DLBCL, one was HIV positive. We focused our study on ten immunocompetent patients with ST-DLBCL. *Results.* Median age was 71 years (range, 40-86). The male to female ratio was 1.5. All patients presented with a palpable mass which was the cause of the initial consultation. Sarcoma was the presumed initial diagnosis in half of the patients. The soft tissue involved was: the posterior chest wall (n=1), the pectoralis major muscle (n=1), the biceps (n=1), the crow's foot (n=1), the gluteus maximus (n=1), the transverse abdominis (n=1), the thigh (rectus femoris n=1, posterior chamber n=1), the gastrocnemius muscles (n=1) and pelvic soft tissues (n=1). Two patients had a performance status ≥ 2 and none had B symptoms. Ann Arbor staging was as followed: stage IV =5, stage IIE =3, stage IE =2. All had elevated LDH level. Three patients had associated nodal involvement whereas 5 had other extra nodal localisations (bone marrow n=3, pleura n=1, meningeal involvement n=1 and underlying bone involvement n=2). Four patients had low-intermediate IPI risk, whereas others patients had high-intermediate (n=3) and high (n=3) IPI risk. Nine patients received anthracyclin-based chemotherapy (RCHOP n=4; CHOP n=3; RMCOPA n=1, RACVBP followed by high-dose chemotherapy with autologous stem cell transplantation n=1). One patient underwent surgery alone. Seven patients had a complete response, one had a partial response and one had primary refractory disease. One patient died after 2 courses of sepsis. Three patients relapsed, all with soft tissue localisations. At the end of follow-up, 6 patients had persistent complete response, one had progressive disease and 2 had died of disease progression. With a median follow-up of 60 months, the 5-year overall survival (OS) and event-free survival (EFS) were 70% (CI95% [40-89]) and 40% (CI95% [17-69]), respectively. Comparison of outcome of ST-DLBCL and 400 none ST-DLBCL treated in our institution between 1996 and 2008 showed that OS and EFS were similar: 5-year OS and EFS were 67% and 62%, respectively for none ST-DLBCL (P=0.94 for OS, P=0.10 for EFS). *Conclusions.* Primary soft tissue involvement is a rare presentation of DLBCL and can be mistaken for sarcoma. Such clinical presentation should require core needle biopsy to allow appropriate diagnosis and therapeutic management. The IPI score and LDH levels of ST-DLBCL are frequently high, but outcome does not seem to differ from none ST-DLBCL.

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NO DIFFERENCE OF SURVIVAL BETWEEN LOCALIZED AND DISSEMINATED DISEASE IN PATIENTS WITH STAGE II DIFFUSE LARGE B-CELL LYMPHOMA

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Background Treatment approach of patients with stage II diffuse large B-cell lymphoma (DLBCL) is usually different according to tumor extent. Therefore, Stage II DLBCL has been categorized into localized disease (LD; 2 adjacent lymph-node regions involved or organ involvement with involvement of regional lymph-nodes), and disseminated disease (DD; extensive involvement over LD). *Aims.* This study was intended to assess prognostic factors and compare survival outcome between LD and DD in patients stage II DLBCL. *Methods.* We searched lymphoma database of Asan Medical Center, Seoul, Korea, and found 91 patients with stage II DLBCL who diagnosed between January 2000 and October 2007. We performed retrospective analysis of progression-free survival (PFS) and overall survival (OS) according to the clinical factors, such as age, sex, tumor extent, lactate dehydrogenase, performance status, tumor location (i.e., supradiaphragm *vs.* infradiaphragm), primary extranodal disease, treatment method, and chemotherapy regimen. *Results.* Median age (range) was 53 (18-83) year-old, and 56 (61.5%) patients were male. Sixty-two (68.1%) patients received chemotherapy and 29 (31.9%) patients received combined chemotherapy and radiotherapy. In terms of tumor extent, 59 (64.8%) patients were LD and 32 (35.2%) were DD. CHOP was administered in 39 (42.9%) patients and R-CHOP was in 52 (57.1%). Median cycle (range) of chemotherapy was 4 (1-8). With 55 months of median follow-up, 5 year-rates of PFS and OS were 71% and 75%, respectively. There was no difference of survival outcome (P=0.46 in PFS, P=0.30 in OS) between LD and DD. The results were consistent if LD and DD were compared in patients with each treatment modality (chemotherapy or combined chemotherapy and radiotherapy) and chemotherapy regimen (CHOP or R-CHOP). Young age (≤60 years) was related to better survival outcomes (P=0.02 in PFS, P=0.02 in OS), however other clinical factors were not associated with survival. *Summary/Conclusions.* In patients with stage II DLBCL, survival outcomes were not different whether tumor is localized or disseminated. Although old age was associated with poor survival outcomes, any other clinical factors fail to show prognostic information. To provide personalized care for stage II DLBCL, further studies are warranted to investigate new prognostic or predictive factors.

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CLINICAL FEATURES AND RESPONSE TO CHEMOTHERAPY OF CHRONIC ACTIVE EPSTEIN-BARR VIRUS INFECTION IN ADULTHOOD: A RETROSPECTIVE ANALYSIS

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Background. Chronic active Epstein-Barr virus (EBV) infection (CAEBV) is a rare disease accompanied by persistent infectious mononucleosis-like syndrome associated with high titers of antibodies. In these patients, T or NK cells are EBV-infected and proliferate clonally. Because most of these patients are children, severe CAEBV with clonally proliferating T cells was named EBV-positive T-cell lymphoproliferative disorders (EBV-TLPD) of childhood according to the WHO classification revised in 2008. However, adult-onset CAEBV has been reported. In addition, EBV can also infect NK cells, causing clonal expansion resulting in CAEBV. Placement of adult-onset or NK cell-infected CAEBV in the WHO classification, and its recommended treatment has neither been discussed nor reviewed. *Aim.* The aim was to clarify the clinical features and response to chemotherapy for adult CAEBV. *Methods.* We reviewed patients who were treated in our hospital according to the strategy originally arranged

for children consisting of cyclosporin A and prednisolone (CP) followed by CHOP and the Capizzi regimen (Int J Hematol, 82:437, 2005). Diagnosis was made according to the criteria suggested by Okano *et al*. (Am J Hematol, 80:64,2005). To detect EBV-infected cells, T and NK cells were separated using magnetic beads from peripheral blood mononuclear cells of CAEBV patients. EBV-DNA was quantified using a real-time quantitative PCR assay. The clonality of EBV was examined by southern blotting using a terminal repeat probe. Written informed consent was obtained from each patient. *Results.* We diagnosed and treated 4 adult CAEBV patients aged 24-48 years at the start of treatment. Infected cells in all patients were T cells. Monoclonal expansion was confirmed in all patients. Three patients were adult-onset, and duration from the onset of disease to treatment initiation was 4-18 months. The onset of disease in the youngest patient was at 7 years of age, while the treatment began at 24 years of age. CP followed by CHOP was given to all. Three patients demonstrated no effects, and only 1, CD4-type patient, achieved 1-log reduction of EBV-DNA in peripheral blood. Grade 4 neutropenia was developed in all patients. The Capizzi regimen was administered to 3 patients. Shortly after starting the regimen, grade 4 non-hematological events occurred in all patients, which led to the discontinuation of cytarabine. Next we reviewed 36 adult-onset CAEBV, adding 33 reported patients searched and selected from PubMed to the present 3 patients, in order to clarify clinical features. T cells were most commonly infected (32/36), whereas infection of T and NK cell types were equally common in child-onset CAEV. Monoclonality was confirmed in 18 patients. Clinical course in 33 patients was documented, and 15 (45.5%) died within 12 months. The average duration from treatment initiation to death was 22.6 days for NK cell-infected type and 8.6 months for T cell-infected type, whereas child-onset CAEV could be observed for 12 to 336 months. *Conclusion.* Adult-onset CAEBV might be aggressive and chemoresistant and has different clinical features from that of child cases. Cases should be accumulated to clarify clinical features and to establish optimal treatment.

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RESULTS OF R-CODOX-M/IVAC IN BURKITT-LIKE NHL AND AGGRESSIVE CELL-B NHL

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Background. Poor prognosis diffuse large B-cell lymphoma (DLBCL) represents 50% of all DLBCL with overall cure rates ranging from 50-60% with dose-dense immunochemotherapy regimens such as R-CHOP. CODOX-M/ IVAC, this regimen is very intensive and was introduced by Magrath and colleagues at the National Cancer Institute, USA to treat Burkitt's lymphoma. Early results in this rare condition for both children and adults were very promising and the regimen has been adopted by many centers for the treatment of the most aggressive, poor risk high grade non-Hodgkin's lymphomas. The search for new treatment strategies in order to improve the survival rate, it should be continue. Therefore, we compared the results of patients treated with R-CHOP and those patients that had received therapy with CODOX-M/ IVAC regimen. *Material and methods.* We reported our experience employing CODOX-M/IVAC-R regimen for patients with poor prognosis diffuse large B-cell lymphoma (DLBCL), burkitt-like NHL and grade 3 follicular NHL. Thirty-nine patients, aged 13-91-years (median 60.3), with an age-adjusted International Prognostic Index (IPI) of 0 or 5, were enrolled since 2003. Group A: It included at 25 patients treated with R-CHOP, aged 20-91 (median 66.16), aaIIPI >3 was presented in 16% patients. Number of cycles administered ranged between 1-8 (median 5.2), 32% (8 patients) had an III-IV stage and aaIPI of 2-4. Overall response was 87.5%, 8 patients with poor prognosis (75%) achieved a complete remission (CR), one patient (12-5%) achieved a partial remission, and one patient (12.5%) died during induction because of bacteriemia. One patient died within 6-months due to infection. None patient had progressed at moment. Grade 3-4 neutropenia developed in 52% of cycles and neutropenic fever in 14% of cycles (51% of patients). Group B: It included at 14 patients treated with R-CODOX-M/IVAC, 13 patients treated in first line, and one patient treated after he had progressed with 2 cycles of R-EPOCH. Patients were aged 13-73 (median 48.78). Number of cycles administered ranged between 1-5 (median 3.7), 27.1% (8 patients) had an III-IV stage and aaIPI of 0-5 (median 2.4), 67% patients had an IPI>3. Overall, 12 patients (85.7%) achieved a complete remission (CR), and two patients (14.3%) died during induction because of bacteriemia. Two patients have relapsed at nine months since diagnosis. One of them patients relapsed and died within 9-months due to infection. Grade 4 neutropenia developed in 100% of cycles and neutropenic fever in 100% of cycles. *Conclusions.* 1) Overall response is similar in both groups (87.5%), with a higher CR rate in the group of patients treated with R-CODOX-M (87.5 *vs.* 75%). 2) The toxicity profile is poorer in the group of patients treated with R-CODOX-M, with a mortality rate greater at induction (first cycle) 14.5 *vs.* 12.3%). We should remember that this group was younger (48.8 *vs.* 60.3) and they had received loss number of cycles (3.7 *vs.* 5.2).

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FRONTLINE ANTIVIRAL THERAPY IN A SERIES OF HCV-RELATED LOW GRADE NON-HODGKIN LYMPHOMA

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Background. The association between HCV infection and type II crioglobulinemia or B-cell non-Hodgkin lymphoma (NHL) suggested its role in clonal B cell proliferation. The regression of NHL after antiviral treatment could be an indirect evidence of this pathogenetic hypothesis. *Aim.* We have treated 5 patients, affected by low grade B-cell NHL (LG-NHL), with different clinical presentations, with antiviral therapy alone with pegylated interferon (PegIFN) and ribavirin (Rbv) in order to evaluate the hematological response in respect of viral response. *Methods.* From 2005 to 2009, 5 patients, affected by LG- NHL at diagnosis have been treated by only antiviral therapy with PegIFN and Rbv (PegIFNα2a 180 mcg weekly, Rbv 800-1000 mg daily) for 6 months (4 pts) or 12 months (1 pt). M/F ratio was 4/1; the mean age was 57.2 years (range 51-73). HCV infection was diagnosed by means of HCV-RNA poliymerase chain reaction (3 cases with genotype 2 and 2 with 1b), before in 4 pts and concomitant in 1 patient, at lymphoma diagnosis. Only one patient has already received other combinations of antiviral therapy in the past. The study included 2 marginal zone lymphoma (MZL), 2 not otherwise specified LG-NHL and 1 lymphplasmacytic lymphoma/ Waldenstrom macroglobulinemia (LPL). The two marginal zone lymphomas showed bone marrow involvement, one with abdominal nodes, bilateral orbital mass and sub cutaneous lesions, the other with splenomegaly, thoracic and abdominal nodes and peripheral B cell clone. The two LG-NHL showed in one case only multiple liver lesions, in the other the patient presented with splenomegaly, a modest bone marrow involvement and a peripheral B cell clone (CD5, CD10 and CD43 negative). The case of LPL presented with splenomegaly, bone marrow involvement and carried a serum monoclonal component IgMk. No patients presented nor B symptom neither bulky disease. *Results.* All five patients completed the planned treatment course. Sustained virologic response (SVR) was achieved in four patients. Hematologic re-staging at the end of antiviral therapy, demonstrated that, among the four patients that gained a SVR, 3 had a CR and one had a PR. The patient that obtained only a reduction of viremia was in hematologic PR. The treatment was well tolerated; only one patients presented a hematologic toxicity grade I WHO, and needed erithropoietine support. After a mean follow up of 14.6 months from the end of therapy (range 1-27), three patients are still in CR and maintain SVR, two patients are in PR; interestingly one patient in SVR, presents a progressive reduction of monoclonal component and splenomegaly. *Conclusion.* Although the limited number of patients involved, the study demonstrates that antiviral therapy could be considered as frontline therapeutic option in a subset of HCV-related LG-NHL, confirming the important role of HCV chronic stimulation in lymphomagenesis. The relationship between SVR and hematologic response is impressive and, in 1 patient, the quality of PR improved during the follow-up.

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USEFULNESS OF POSITRON EMISSION TOMOGRAPHY/COMPUTER TOMOGRAPHY (PET/CT) IN PATIENTS WITH BURKITT OR BURKITT-LIKE LYMPHOMA/LEUKEMIA. PRELIMINARY DATA FROM A SINGLE INSTITUTION

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Background. Positron emission tomograph /Computer Tomography is a non invasive imaging tool increasingly used in the management of lymphoma. In the setting of Burkitts' Lymphoma/Leukemia (BL) a study supporting the usefulness of PET/CT at diagnosis has been recently reported (Zeng 2009). No data on the usefulness of PET/CT during or after treatment are available. The GMALL B-ALL/NHL 2002 treatment protocol is reported as one of the most effective treatments of BL with a high cure rate. However a proportion of patients still relapses or fails treatment. *Aims.* To analyze the role of PET/CT integrated into the GMALL B-ALL/NHL 2002 protocol in order to optimize management of BL or diffuse large B cell lymphoma (DLBLC) with high proliferation index. *Patients and methods.* From August 2004 to June 2009 29 patients with acute mature B Burkitts' Leukemia (7), Burkitts' Lymphoma (16) and DLBCL (6) were treated at our institution according to the GMALL B-ALL/NHL 2002 protocol. PET-CT was performed in 15 patients (4 with mature B-ALL, 8 with BL and 3 with DLBCL). PET/CT imaging was available at diagnosis in 12/15 patients, during treatment in 6 patients, and in 11 patients at the end of treatment, since 4 patients (with positive PET/CT at diagnosis) died of infection during the first or second block of treatment and could therefore not been evaluated. *Results.* In 8 patients (3 BL, 3 mature B-ALL, 2 DLBCL) PET/CT was strongly positive at diagnosis and proved negative at the end of treatment; all these patients are in continuous complete remission (CCR) with a median follow up of 407 days. Six patients underwent PET/CT scan during treatment: 3 were proved negative, 2 doubtful (negative after subsequent treatment) and one positive. In one patient positive after 2 blocks, PET/CT scan demonstrated progression of disease at the end of treatment and the patient died one month later. In two patients undergoing PET/CT only at the end of therapy, no metabolic uptake of 18F-FDG was detected and the patients are currently in CCR. Therefore PET/CT scan performed at the end of protocol had a 100% accuracy in predicting one year disease free survival. *Conclusions.* Despite the low number of patients of this retrospective study, negative PET at the end of treatment according to GMALL B-ALL/NHL 2002 protocol is associated with sustained CCR and probably cure, given the long duration of follow-up. On the other hand in patients with a positive scan further treatment should be considered mandatory. The enrollment of more patients - currently ongoing - is needed to confirm this data and the role of PET/CT after 2 blocks in order to modulate the intensity of treatment is under investigation.

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UTILITY OF 18F-FDG-PET IN MARGINAL ZONE LYMPHOMA

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Background. WHO classification distinguished three different MZL types derived from the marginal zone of B-cell follicles: splenic marginal zone lymphoma, MALT lymphoma and nodal marginal zone lymphoma. According to recent reports imaging with 18F-FDG-PET has a limited role in the diagnostic workup of patients with MZL as involvement is less reliable at detecting marginal zone lymphoma, particularly in the case of extranodal MZL. *Materials and methods.* We retrospective studied 21 patients with MZL who had undergone 18F-FDG-PET at the time of diagnosis. Whole-body 18F-FDG-PET scans were performed on a PET scanner 60 min after intravenous injection of 428.23MBq of 18F-FDG. *Results.* The group included 21 patients, with an age range of 40-79 years (average, 62 years), 52% woman. Histological reassessment of biopsy specimens confirmed the diagnosis of MALT MZL in 12 patients, while a diagnosis of splenic MZL was verified in 7 patients. Two patients presented with peripheral blood disease. Common sites of MALT lymphoma were the oral cavity (2), lung (1), orbit (1), lachrymal gland (1), cavum (3), and small bowel (1). Forty-three percent of the patients had localized disease (stages I and II), and 57% had bone marrow involvement at presentation. Nineteen percent presented various abnormalities in the karyotype. B symptoms were present in only four patients (19%). Most patients were categorized as low or low-intermediate risk group by international prognostic index (IPI). Two patients associated hepatitis C virus infection. 18F-FDG PET detected disease in 52% of patients, with a medium SUVmáx of 4,48 (1,42-10). Five patients with splenic MZL (71%) showed a positive 18F-FDG-PET at diagnosis, with a medium SUVmáx uptake in the spleen of 3,26. We did not find statistically significant differences comparing the results of 18F-FDG-PET between splenic MZL and MALT lymphoma. 18F-FDG-PET detected 29 disease sites (average 1.4 disease sites per patient, range 0-6), 15 of these lesions were lymph node regions and 8 were extranodal lesions. 18F-FDG-PET detected bone marrow disease in only 2 of 13 patients with bone marrow involvement (15%). Fifty percent of the patients achieved complete remission (CR) after the initial therapy, that includes Rituximab and fludarabine in most of them. The median follow-up was 1 year (range 0-3). *Conclusions.* These data suggest that 18F-FDG-PET is a useful tool for initial staging in patients with MZL. Its sensitivity depends on disease location and stage at initial diagnosis. The high number of patients in whom PET did not detect bone marrow involvement suggest that PET is complementary to bone marrow aspiration and trepination in this group of patients.

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UPFRONT TREATMENT OF MANTLE CELL LYMPHOMA BY SEQUENTIAL HIGH DOSE IMMUNOCHEMOTHERAPY SUPPORTED BY *IN VIVO*-PURGED STEM CELL DOUBLE AUTOLOGOUS TRANSPLANTATION: A SINGLE INSTITUTION EXPERIENCE

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Background. Mantle Cell Lymphoma (MCL) is characterized by a good response rate to conventional anthracycline-based regimens but long-lasting remissions are sporadic. *Aims.* we retrospectively analyzed the outcome of 15 MCL patients upfront treated with sequential high dose immunochemotherapy followed by double autologous stem cell transplant (ASCT). Patients and *Methods.* from 2000 to 2009, 15 MCL patients, eligible for ASCT, have been consecutively treated as follow: a) standard dose phase: APO: doxorubicin, 75 mg/sqm i.v., day 1; prednisone, 60 mg/sqm orally, day 1 to 5 and day 9 to 12; vincristine, 1.4 mg/sqm i.v., day 1 and 8; DHAP: cisplatin 70 mg/sqm, day 1; cytarabin 1500 mg/sqm i.v., days 2-3; dexametasone 40 mg i.v., days 1 to 3; b) rituximab high dose sequence: high dose cyclophosphamide (CTX 5 g/sqm) and high dose cytarabine (Ara-C 2 g/sqm every 12 for 6 consecutive days) followed by leukapheresis; c) high dose melphalan (180 mg/sqm) and high dose mitoxantrone plus melphalan (60 mg/sqm and 180 mg/sqm, respectively) followed by PSC infusion. Rituximab (375 mg/sqm) was infused twice after CTX, cytarabine and double autologous transplantation (modified from Gianni *et al.*, Blood, 102, 749, 2003). All patients (8 female and 7 male) had a histological diagnosis of MCL according to WHO classification criteria; molecular rearrangement of bcl-1 locus was detected by PCR in the bone marrow of 7 patients. The median age at diagnosis was 57 years (range 37-68); 13 patients were in stage IV and 2 in stage III; 2 patients had bulky disease at presentation. Four patients were in overt leukemic phase and 2 had extranodal localization. According to MIPI score, 10 patients were classified as low risk (67%), 3 as intermediate risk (2%) and 2 as high risk (1%). Double transplant was performed in all but 2 patients (one for refusal and one because second procedure is currently ongoing). *Results.* The standard dose phase, which included a median number of 4 cycles (range 3-5), was generally well tolerated, with only one patient experiencing tumor lysis syndrome. After induction, clinical CR was achieved in four patients. PBSC were successfully collected after both CTX/rituximab ($1.8\text{-}9.7\times10^6$/Kg) and Ara-C/rituximab (7.1- 40.0×10^6/Kg) cycles. At the end of these phases, 5 patients were in CR while 10 were in PR. Following transplants, median times to ANC >500/μL were 11 days (range 10-14) in both the procedures, whereas median times to platelet recovery (>50000/μL) were 18 days (range 10-172) after the first transplant and 23 days (range 11-298) after the second one. Most frequent complications were FUO and grade III-IV mucositis, especially following the second transplant. After a median follow-up of 28 months (range 13-105), 11 patients (73%) were alive, whereas 4 died from disease progression. With regard to disease status, 11 patients were in CR and 4 patients were in PR. Both overall and disease free survival at 7-years were 70%, respectively. *Conclusions.* Our results confirmed that in MCL the use of sequential high dose immunochemotherapy followed by double autologous transplantation associates to high remission rates with long-term survival.

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HIGH GRADE B-NHL IN FRAIL PATIENTS: MANAGEMENT AND RESULTS OF A SINGLE INSTITUTION

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The fate of those patients with high grade NHL, who do not enter in specific protocols because the very old age and the presence of comorbidities, is generally not known or not extensively dealt with. In fact a proportion of patients with NHL of about 20% are so called frail because the age or clinical problems out of the lymphoma. We present our experience on 68 patients with B-HGNHL defined frail and observed over 4 years, since 2002 to 2006. The median age was 68y (61-81) with a prevalence of males (31/27). Symptoms B were present in 35(53%) and advanced stage (III-IV) was recognized in 50(73%); histology was DLCBL in 63(94%) and MCL in 5. PS was >2 in 62(93%). Comorbidities were inclusive of heart problems (52%), diabetes (28%), renal failure (12%), liver elevation of enzymes or liver failure (18%); 65 pts (96%) had almost 2 comorbidities. The therapy was patient related by avoiding Antracyclines in case of myocardial problems, avoiding glucocorticoids in unregulated diabetes and avoiding intensive or great associations in those with renal or liver falure. Schemes included VNCOP-B (16 pts), CHOP (7pts), CHOP/R (9pts), CHOP-like with liposomal Doxorubicin and Rituximab (5 pts), CVP/R (13 pts), Vincristine and Cyclophosphamide low dose (18 pts). 70% of pts reduced or discontinued the therapy and 30% stopped definitely the treatment. Supportive care consisted of transfusion or growth factors as needed. The overall response rate inclusive of CR and good PR was 30% in those patients who completed the therapy not inclusive of the Antracyclin, with rituximab or not, and 55% in those inclusive of Antracyclin, either liposomal or not, and Rituximab; other patients treated by alternative schemes had mild or transient responses. Side effects diabetes disregulation (98%), infections (13%), heart falure (15%), renal falure (20%). Deaths due to therapy were 2 in those treated by Antracyclin. The fate of patients who obtained remission is to maintain CR in 60% of those who did Rituximab and Antracyclin and 20% of those who did not. The cause of death was the progression of lymphoma in 80% of those who did not obtained remission and comorbidity related in 20% of the same patients. The relapsed patients had mostly a progression of disease. We conclude that the therapy of frail patients with HG-BNHL should include Antracyclin (liposomal?) and Rituximab in order to obtain more and durable remissions, although the risk of related deaths is increased with this approach.

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CLINICAL CHARACTERISTICS AND THERAPY RESPONSE OF PRIMARY CENTRAL NERVOUS SYSTEM LYMPHOMA (PCNSL) PATIENTS TREATED WITH DE ANGELIS REGIMEN: A SINGLE INSTITUTION EXPERIENCE

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Background. PCNSL is a rare tumor, defined as a non-Hodgkins lymphoma that is confined to the craniospinal axis without systemic involvement. PCNSL accounts for 1% to 2% of all lymphomas and less than 5% of all primary CNS tumors. The outlook has improved but remains generally poor. *Aim.* To compare clinical characteristics in pts with PCNSL and their response to De Angelis regimen (MTX 1 g/m^2 IV d1, d8; 6 doses of intrathecal MTX 12 mg; Dexamethasone 16 mg IV d1-d15, radiotherapy (RT)- 40 Gy to the whole brain + 14 Gy boost to the tumor; after RT: Cytarabin 3 g/m^2 IV d1, d2, d22, d23). *Methods.* 17 pts with PCNSL diagnosed and treated at the Institute of Hematology, Clinical Centre of Serbia, in the period of 1994-2009, were analyzed. The diagnosis was based on histological and immunohistochemical analysis of tumor biopsy. Tumor histology was diffuse large B- cell lymphoma in all our pts. *Results.* We analyzed 17 pts, 12 males and 5 females, median age was 53 (range 27-66 yrs). All of our pts were immunocompetent, there was no evidence of HIV infection. Most pts had symptoms suggestive of an intracranial mass lesion (headaches, personality changes) and less common seizures. Response to De Angelis regimen: 3 pts CR, 6 pts PR, 8 pts (3 pts were older than 60 yrs, 4 pts had ECOG performance status 3 and greater and elevated serum LDH) didn't respond to the therapy and died within 6 months after the diagnosis was established. In responders group 6 pts are alive, 1 pt died in first relapse 4 months after completing De Angelis regimen, 2 pts in first relapse (after 17 months and 27 months respectively) were treated with salvage chemotherapy, achieved secondary remission lasting 38 and 7 months, and both died within 17 months after second relapse. *Conclusion.* PCNSL pts have generally poor outcome and the optimum chemotherapy and radiotherapy strategy has yet to be established.

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IMMUNOCHEMOTHERAPY VERSUS CHEMOTHERAPY IN 90 NEWLY DIAGNOSED PATIENTS WITH FOLLICULAR LYMPHOMA: A SINGLE INSTITUTION STUDY

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Background. Follicular lymphoma (FL) is the most common type of low grade Non Hodgkin Lymphoma. In past few years the great improvement in treatment of follicular lymphoma patients was achieved by introducing immunochemotherapy as the standard first line therapy. All widely accepted prognostic indices such as Follicular Lymphoma International Prognostic Index (FLIPI), Italian Lymphoma Intergroup (ILI) and, for immunochemotherapy treated patients recently established FLIPI 2, identify different risk groups of patients, but great heterogenity in the outcome within these groups still persists. *Aims.* The aim of this study was to compare the outcome of FL patients treated with immunochemotherapy and chemotherapy. The second aim was to identify high risk patients regardless to received frontline therapy. *Methods.* In this retrospective study were included 90 newly diagnosed FL patients treated in the Clinic for Hematology Clinical Centre of Serbia from April 2000 until March 2006. 60 patients were treated with chemotherapy and 30 with immunochemotherapy. Initial FLIPI, ECOG performance status (ECOG PS), ESR and the presence of Bulky tumor (tu mass >7 cm) were determined in all patients. *Results.* Patients treated with immunochemotherapy had signifficantly longer event free survival (EFS) compared to patients treated with chemotherapy (log rank, P=0.028) and there was no difference in overal survival (log rank, P=0.075), but there is a trend toward better survival in patients treated with immunochemotherapy. 52 patients had high risk according to FLIPI, 25 intermediate and 13 low risk. All the patients with low and intermediate risk had high tumor burden according to GELF 94 criteria, predominantly B symptoms and high tumor burden. Bulky disease was present in 29 patients (in 18 treated with chemotherapy and 11 treated with immunochmeotherapy), ESR>30 mm/1h had 48 pts (32 treated with chemotherapy and 16 treated with immunochmeotherapy) and ECOG PS>2 had 26 patients (18 treated with chemotherapy and 8 treated with immunochemotherapy). FLIPI and Bulky disease didn't have prognostic impact on survival in both groups, but there was a trend of worse survival in FLIPI high risk patients regardless to treatment and better survival in FLIPI high risk patients and patients with Bulky disease treated with immunochemotherapy. The patients treated with chemotherapy with ESR>30 mm/1h had signifficantly worse outcome than the patients who didn't (log rank, P=0.002), but this was not recorded in group of patients treated with immunochemotherapy (log rank, P=0.957). The patients treated with immunochemotherapy with ECOG PS>2 had signifficantly worse outcome (log rank, P=0.02), but it was not the case in group of patients treated with chemotherapy (log rank, P=0.497). In patients with ECOG PS>2 there was no difference in outcome regardless to initial treatment, chemotherapy *vs.* immunochemotherapy (log rank, P=0.461). *Conclusions.* Immunochemotherapy improved outcome in patients with newly diagnosed follicular lymphoma and it is overcoming the influence on outcome of ESR recorded in patients treated with chemotherapy. Still, there is a subgroup of patients (ECOG PS>2) that doesn't have a benefit from immunochemotherapy and furhter investigations are needed to define appropriate therapeutic approach to these patients.

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NON-HODGKIN LYMPHOMA WITH SOFT TISSUE LESION

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Introduction. Soft tissue involvement accounts for 5,2% of all non-Hodgkin lymphoma (NHL). The aim of our study was to describe the clinicopathologic characteristics of patients with soft tissue lesion. *Methods.* In N.N. Blokhin Russian Cancer Research Center of the Russian Academy of Medical Science from 1983 to 2007 years 582 patients (pts)

with NHL were observed; 73 (12%) of them had a lesion of soft tissue, diagnosed at various time of a clinical course. The median age was 52 years (range 16-81). Only in 9\73 (12%) cases the lesion of soft tissue had been diagnosed at the first reference of the patient. *Results.* B-cell phenotype of a tumor had 70 from 73 patients, only 3 patients (4 %) had peripheral T-cell lymphoma, not specified. The frequency of various variants of B-lymphomas with soft tissue lesion is presented in Table. According to the International Prognostic Index (IPI) 89% of patients at the primary diagnostics of lymphoma had made a group of the high-intermediate/high risk, 87% of patients had the advanced stage of disease. The majority of patients had more than 1 extranodal site. B-symptoms had 31(44%). Median time from primary diagnostics to soft tissue involvement was 6 month. At 66 from 70 patients soft tissue involvement was a part of relapse/progression and only 4 patients had isolated soft tissue lesion. All patients received treatment according to the morphological variant of NHL, therefore treatment was quite heterogeneous. The median progression-free survival was 5 month, median overall survival - 23 month. *Conclusions.* Last time soft tissue involvement in patients with extranodal lymphomas in connection with growth of disease draws special attention of researchers. Involving of soft tissue in tumoral process is more often diagnosed at later time of development of disease and can be associated with high IPI score (3-5). The majority of NHL with soft tissue lesion had the B-cell origin and concern one of the variants of large cell lymphomas. The treatment strategy in patients with soft tissue lesion because of the listed above reasons need to be corrected.

Table. Distribution of pts according to B-NHL variants.

Variant of B-NHL	Abs.	%%
1. Diffuse large B-cell lymphoma	41	59%
2. Primary Mediastinal (Thymic) Large B Cell Lymphoma	4	6%
3. Burkitt lymphoma	9	13%
4. Follicular lymphoma		
Grade I-II	11	15%
Grade III	2	3%
5. Mantle cell lymphoma	3	4%
TOTAL	70	100%

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PRIMARY B AND T CELL NON HODGKIN'S LYMPHOMA (NHL) OF THE GASTROINTESTINAL (GI) TRACT: COMPARATIVE OUTCOMES FOLLOWING ANTHRACYCLINE BASED CHEMOTHERAPY

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Background. Anthracycline based chemotherapy is the treatment of choice for aggressive primary lymphomas of the GI tract. We report outcomes of 61 patients with primary GI NHL treated over 18 years. *Aim.* To establish the outcome of GI lymphomas, at our institution, according histological subtype. *Methods.* Cases were identified from the histology database and records reviewed. *Results.* There were 34 (56%) males and 27 (44%) females. The median age at diagnosis was 60 (15-83). 43 (70%) were diffuse large B cell lymphoma (DLBCL), 10 (16%) were T-cell and 8 (14%) were mucosa-associated lymphoid tissue tumor (MALT). The 8 patients with MALT were treated with single agent chemotherapy; 7 (88%) are alive at median follow up of 8.5 years (2-16). Of the aggressive lymphomas (53 DLBCL and T cell), all patients with T cell lymphoma had small bowel as primary site and histological evidence of coeliac associated enteropathy, even in the absence of known coeliac disease. Primary sites of DLBCL were stomach in 30 patients (70%), small bowel in 8 (19%) and colon in 5 (11%). 34 patients underwent surgery at diagnosis due to acute presentation with perforation, obstruction or bleeding, or to obtain histology. Following confirmed diagnosis, 51 patients received anthracycline-based chemotherapy (CHOP/ CHOP-R/ M-BACOD). 2 patients with T-cell lymphoma presented with perforation, had surgery only and died of rapid disease progression. Of the 53 patients with aggressive NHL, 33 (62%) remain alive and disease free at median follow up of 7 years (1-18). 31 (72%) with DLBCL are alive and disease free, 4 deaths in this group were not related to cancer or treatment. 2 (20%) of the T-cell lymphomas are alive and disease free, all deaths in this group were from progressive disease. *Conclusion.* Patients with aggressive primary B cell GI NHL have >70% survival following anthracycline based chemotherapy. However, in contrast, coeliac enteropathy associated T cell lymphomas present with rapidly progressive disease and have a poor prognosis. Novel therapeutic approaches are required to improve outcome in this group.

1600

CIRCULATING CYTOKINE LEVELS IN PATIENTS WITH NON-HODGKIN LYMPHOMA AND OTHER LYMPHOPROLIFERATIVE DISORDERS

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Background. Is it known about important part of different cytokines in pathogenesis and course of lymphoproliferative diseases. But the going data are not absolute and entire. *Aims.* The aim of the present study was an evaluation of circulating cytokine levels in patients with Non-Hodgkin lymphoma and other lymphoproliferative disorders. *Methods.* The study included 20 patients with Non-Hodgkin lymphoma (NHL), 10 patients with Hodgkin lymphoma (HL), and 10 patients with multiple myeloma (MM) (18 males, 22 females, mean age is 55,43±2,41). Serum concentrations of cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, IFN-α, IFN-γ, IL-1RA, and antibodies to IFN-α) were studied with enzyme-linked immunoassay (Vector-Best, Novosibirsk, Russia). Written informed consent was obtained from each patient. *Results.* Increasing levels of pro-inflammatory cytokines IL-6, IL-8, IFN-γ were found. The mean level of cytokines did not differ between NHL, HL and MM groups with the exception of IL-10 which concentration was the highest in NHL and the lowest in MM (37.7±8.5 pg/mL and 6.1±5.4 pg/mL, respectively, P=0.013). A significant correlation between the concentration of pro-inflammatory cytokines: IFN-γ and IL-6 (r=0.51, P=0.001), IFN-γ and IL-8 (r=0.39; P=0.014), IL-6 and IL-8 (r = 0.37; P=0.019), as well as between the level of anti-inflammatory cytokine IL-10 and the levels of pro-inflammatory cytokines IL-8 (r=0.39, P=0.015), IL-6 (r=0.46, P=0.003), and IFN-γ (r=0.65, P<0.001) were revealed. Therefore, there is an interdependent increasing concentrations of cytokines IL-6, IL-8, IFN-γ, and the resulting increase in the level of anti-inflammatory cytokine IL-10. Symptoms of tumor activity were not associated with normal levels of IL-10 and IL-6 (P=0.016). Correlation was found between the level of IL-6 and the level of C-reactive protein (r=0.85, P<0.001), and between the increase in IFN-γ and abnormal weight loss (r=0.34, P=0.039). Hemoglobin level <80 g/l was correlated with increased IL-8 level (r=0.38, P=0.017). Infiltration of bone marrow with tumor cells in NHL was associated with increased concentration of IFN-γ (P=0.036) and IL-10 (P=0.026). *Summary.* In conclusion, among the cytokine panel studied, increased levels of pro-inflammatory cytokines IL-6, IL-8, IFN-γ and anti-inflammatory cytokine IL-10 were observed. This increase was associated with clinical and laboratory symptoms that characterize the aggressive course of disease. Additionally, anemia was associated with increased production of IL-8.

1601

HIGH INCIDENCE OF HEPATITIS B OR C VIRUS INFECTION IN LYMPHOMAS

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Introduction. Several reports for different countries have shown that patients with lymphoma have a higher prevalence of HCV or HBV infections. The infection rates were different even in virus endemic areas as Balkan countries, including Romania. However, an association between the virus infection and lymphoma should be evaluated and discussed with the background of virus prevalence in the comunity. This study showed the prevalence of HBV or HCV infection status in lymphoma at patients in the Oltenia county of Romania. *Methods and results.* the study was conduted in Oltenia county (Romania). This study included 321 newly diagnosed patients with lymphomas admitted in the Clinic of Hematology from Craiova and 298 non-lymphomas control patients enrolled from January 2001 to December 2009. We evaluated the prevalence of HBV or HCV infections in both groups of patients and compared the clinical-pathological characteristics HBV or HCV positve and its negative lymphoma cases (17 of 321, 11,1%) than control group (2,6%, P<0,001).The higher prevalence was observed in both sexes and especially diffuse large B-cell lymphoma; compared with the HBV negative lymphoma group, the positive group displayed more liver or spleen involvement (P=0,001). The rate of HCV infection was higher

than those in the control group in HCV infected cases, more liver involvement was indicated (P<0,003). *Conclusions.* the present study, so far the largest trial of incidence of HBV or HCV infections at lymphoma patients demonstrated that patients with lymphoma in Romania had higher prevalence of HBV or HCV infection. This appears to be a possible role of HBV or HCV infection in the induction of malignant transformations, resulting in the development of lymphoma.

1602

POORER PROGNOSIS OF INTERMEDIATE BL/DLBCL COMPARED TO BURKITT LYMPHOMA IN RITUXIMAB ERA: A RETROSPECTIVE ANALYSIS WITH CLINICAL AND PATHOLOGICAL FEATURES

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Background and aims. The distinction of Burkitt lymphoma (BL) and diffuse large B-cell lymphoma (DLBCL) is critical for the therapeutic strategy. But there are some population with features of both BL and DLBCL. Such cases are now categorized into intermediate BL/DLBCL in 4th edition of WHO classification. Intermediate BL/DLBCL often has aggressive clinical courses, but at the present time optimal therapy was not established to this entity. To evaluate the clinical outcomes of intermediate BL/DLBCL in rituximab era, we conducted a retrospective cohort study of intermediate BL/DLBCL, BL, and DLBCL. *Methods.* All DLBCL and BL newly diagnosed and treated at our hospital after Dec 2003 (rituximab era in Japan) were serially registerd. Distinction of BL and intermediate BL/DLBCL was mainly based on morphological features, including immunohistochemistry (bcl2 expression and MIB1 index). Genetic information was not available for all patients. Overall survival (OS) was assessed using the Kaplan-Meier method, and the groups (BL and intermediate BL/DLBCL) were compared using the log-rank test. *Results.* Eleven BL and 10 intermediate BL/DLBCLs were included in this study. All patients diagnosed as BL received high intensity regimen (i.e. CODOX-M/IVAC, HyprCVAD/MA) without rituximab except for one case. Five out of 10 intermediate BL/DLBCL patients were treated by R-CHOP based regimen, and others were treated by high intensity regimen mostly in combination with rituximab. Median age and the IPI risk group between these disease entities were not statistically different. The 3 years OS rate was 79.6% in BL and 32.8% in intermediate BL/DLBCL. The favorable clinical outcome was shown in BL rather than intermediate BL/DLBCL, nevertheless the difference was not statistically significant (P=0.126). *Conclusions.* Intermediate BL/DLBCL showed poorer prognosis than Burkitt lymphoma, and the benefit of the high intensity therapy in combination with rituximab remains to be elucidated.

1603

SIX-YEAR EXPERIENCE IN THE TREATMENT OF PRIMARY AGGRESSIVE GASTRIC LYMPHOMAS WITH MODIFIED NHL-BFM-90 PROTOCOL

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Background. CHOP chemotherapy can induce complete responses (CR) in up to 80% of patients with Primary Aggressive Gastric Lymphomas (PAGL) but the presence of adverse prognostic factors (APF) (bulky disease; stage>IE; B-symptoms; elevated LDH; undetected diagnosis of primary gastric Burkitt's lymphoma) decrease the efficacy of this therapy. Optimal therapy of these lymphomas is controversial. *Aim.* efficacy and safety assessment of the modified chemotherapy protocol NHL-BFM-90 (mNHL-BFM-90) in the treatment PAGL with APF. *Methods.* Twenty seven patients PAGL with APF underwent mNHL-BFM-90 between Oct 2003 and Dec 2009. The type of lymphoma was classified according to the WHO classification system. NHL-BFM-90 program (2 courses A and 2 courses B) was modified for primary gastric diffuse large B-cell lymphoma (PGDLBL) in the following way: Doxorubicin (50 mg/m^2) was added on the third day of course A. NHL-BFM-90 program (2 courses A and 2 courses C) was modified for primary gastric Burkitt's lymphoma (PGBL) in the following way: Doxorubicin (50 mg/m^2) was added on the third day of course A. Methotrexate (1 g/m^2 i/v) was added on the first day of course C. *Results.* There were 16 women and 11 men. The mean age at presentation was 45 years (14-73 years), 7 patients were older than 60. All patients had one or more APF. Diagnosis of PGDLBL was proved in 21 and PGBL in 6 cases, accordingly. Translocation t(8;14) was assessed for diagnosis of PGBL. None of the patients received surgical treatment before chemotherapy and consolidating radiotherapy. Only 4 patients were treated by mNHL-BFM-90 with addition of rituximab. Hematologic toxity of grade 3 and 4 was observed in all patients. Severe reversible complication became the reason for subsequent switch to CHOP therapy after 2 courses in 6 cases. All patients achieved complete remissions.The mean follow-up is 33 months (range 2-78). No relapses have been registered so far. *Conclusion.* mNHL-BFM-90 protocol is highly effective in both PGDLBL with AFP and PGBL.

1604

PROGNOSTIC SIGNIFICANCE OF APOPTOSIS-RELATED PROTEINS IN DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL) PATIENTS (PTS) TREATED WITH CHOP-BASED CHEMOTHERAPY

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Background. Chemotherapy-induced apoptosis is one of the most important mechanisms of treatment in DLBCL. *Aims.* We evaluated the prognostic significance of apoptosis-related proteins in DLBCL pts treated with cylophosphamide/doxorubicin/vincristine/prednisone (CHOP) chemotheraoy with or without rituximab (R). *Methods.* Pretreatment tumor biopsy specimens from 30 pts (stage I: 4, II: 14, III: 9, IV: 3 pts) with DLBCL were analyzed for Bcl-2, Bcl-6, Bax and P53 proteins expression by immunohistochemistry. Fifteen pts were treated with CHOP regimen, while 15 pts with R-CHOP. *Results.* Within median follow-up duration of 67 months (28-166months) in survivors, 5-years overall survival (OS) of all pts was 65%. In univariate analysis, high expression of Bcl-2 and Bcl-6 was associated with poor OS in all pts (P=0.032 and 0.019). In R-CHOP group, high expression of Bcl-6 was associated with poor outcome (P=0.007), while high expression of Bcl-2, Bax and P53 was not correlated with pts outcome (P=0.086, P=0.055, and P=0.753). In addition, all apoptosis-related proteins did not show any prognostic significance in CHOP group. In multivariate analysis for all pts, high expression of Bcl-6 was the only significant independent predictor of poor OS (P=0.028). *Conclusions.* High expression of Bcl-6 may be useful for prediction of poor outcome in pts with DLBCL treated with R-CHOP.

1605

EVALUATION OF SAFETY, TOLERABILITY AND ACTIVITY: A REGISTRY FOR TEMSIROLIMUS-TREATED PATIENTS WITH MANTLE-CELL LYMPHOMA (MCL) IN THE USUAL HEALTH CARE SETTING

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Background. Temsirolimus (TEMS), an i.v. mTOR inhibitor, was approved for the treatment of patients with relapsed and refractory MCL in Aug 2009. A pivotal study had demonstrated significantly increased progression free survival with TEMS in relapsed and refractory MCL compared to investigator´s choice mono-chemotherapy (4.8 mo *vs.* 1.9 mo). *Aims.* To better identify the safety profile and efficacy of TEMS during clinical routine, continuous collection of data on pharmacovigilance in the post-approval period e.g. in a non-interventional trial (NIT) is essential. *Methods.* To prospectively evaluate TEMS in the usual health care setting a registry for TEMS-treated patients with advanced renal cell carcinoma (aRCC) has already been initiated in Germany in Jan 2008 (NCT00700258). With regulatory and ethic committee's notification this NIT was amended and extended to include patients with MCL. Primary objective is the evaluation of TEMS´s safety profile. Secondary objectives include efficacy of TEMS as well as the profile, comorbidity and characteristics of patients and sequence of systemic therapies in MCL. Inclusion criteria: histologically confirmed diagnosis of MCL that will be treated with TEMS, written informed consent by the patient prior to data collection. *Results.* 297 pts with aRCC have been included from February 2008 to January 2010. Since the amendment to open the NIT for patients with MCL in November 2009, patients with MCL were also included. The data collection period will last for approx. 5 years. Up to the end of January 2010 88 centers have been initiated and are currently recruiting patients. *Conclusions.* To better understand the safety, tolerability and efficacy of TEMS in the treatment of MCL - a rare tumor entity - in the real-life clinical setting Wyeth started a registry to further evaluate TEMS in the usual health care setting. First results of the MCL-part of this NIT will be presented in June 2010.

1606

AUTOLOGOUS STEM CELL TRANSPLANTATION (ASCT) IN POOR-PROGNOSIS DIFFUSE LARGE-B CELL LYMPHOMA (DLBCL)

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Background. High-dose therapy and ASCT is considered to be an option of treatment to overcome cellular resistance and eradicate minimal disease in patients with non-Hodgkin's lymphoma *Aims.* The purpose of this study was to evaluate the impact on outcome of ASCT in patients with poor prognosis DLBCL. *Methods.* We have retrospectively analyzed 43 patients with DLBCL who underwent high-dose therapy and ASCT between January 1993 and March 2009 in our institution. Information was gathered from clinical records. *Results.* There were 22 (51%) males and 21 (49%) females with a median age of 45 years (range, 14-71 years). At diagnosis, 35 patients (81%) were in stage III-IV, 22 (51%) suffered B-symptoms, bulky disease was present in 20 (46%) and extranodal disease in 25 (58%). LDH value was available in 41 cases and was elevated in 88%. The International Prognosis Index (IPI) was evaluated in 41 and in 22 (54%) was ≥3. Median number of prior chemotherapy regimens was 2 (range, 1-3), and 4 (9%) had received prior radiation as part of induction or as salvage therapy. The median time from diagnosis to ASCT was 8 months (range, 5-36 months). Nineteen (44%) underwent transplantation in first complete remission (CR), 14 of then had received only one prior regimen and underwent ASCT as consolidation (IPI ≥3). Nineteen (44%) were in partial remission at ASCT, and 5 (12%) in second or subsequent remission. Conditioning regimens were carmustine, etoposide, cytarabine, and melphalan (BEAM) in 41 patients, total body irradiation/high-dose cyclophosphamide in 1 patient and bususfan/cyclophosphamide/thiotepa in another. All patients received peripheral blood stem cells. Fifteen patients with bulky disease at diagnosis received involved field irradiation as consolidation after ASCT. The early procedure-related mortality rate was 2,3% (one of 43 patients) and cardiac toxicity has been observed in one patient. At 3 months, the CR rate was 84%, two cases had relapsed and 5 were in progression. At a median follow-up 30 months (range, 1-158 months), the 5-year Kaplan-Meier estimates of probability of overall survival and event free survival are 64% and 62% respectively. Patients who underwent ASCT in first CR as early consolidation because of IPI ≥3 (n=14) had an actuarial 5-year survival rate of 75% compared with 55% for those with resistant/relapsed disease (P=0,04). Twenty-nine patients remain in CR maintained without continued therapy. The 13 cases with relapsed or resistant disease after transplant have received salvage chemotherapy but 12 of them have died. No second malignancies have been detected. *Conclusions.* ASCT is an effective regimen in poor risk DLBCL with low toxicity. Results of ASCT as consolidation in patients with IPI ≥3 in first CR are encouraging but prospective randomized trials are needed.

1607

LONG-TERM SURVIVAL OF MANTLE CELL LYMPHOMA - A SINGLE INSTITUTION EXPERIENCE IN TAIWAN

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Background. Mantle cell lymphoma is a well-defined subtype of B-cell non-Hodgkin lymphoma which is approximately 6% of lymphoid neoplasm. The median survival is 3 to 4 years as literatures reported. Typically in older adults patients with male predominance and patients usually presented with stage IV disease. The response to chemotherapy usually resulted in tumor regression but unmaintained remissions are short. *Methods.* Between March 2003 and August 2009, we treated 38 mantle cell lymphoma patients with various protocols in 17 years. Diagnosis of mantle cell lymphoma is according to the histopathology and flow cytometry with positive for CD20, CD5, and negative for CD23 or cyclin D1(+). Initial treatment regimen includes regular intensity chemotherapy (eg. CHOP, COP, chlorambucil and prednisolone, with or without rituximab) for 32 patients and intensive chemotherapy (eg. HyperCVAD/MA or high dose Ara-C containing chemotherapy) for 6 patients. *Results.* In these 38 patients, the median age on initial presentation is 60.8 years with range between 26 and 77 years of age with male predominance (M/F 29/9). Most patients (76.3%) presented with stage IV disease (29/38). There were 4 patients underwent reduced intensity allogeneic peripheral blood stem cell transplantation as the salvage treatment. The 8-year disease free survival was 15.9% and overall survival rate was 28.4%. All the 6 patients underwent intensive HyperCVAD/MA chemotherapy are alive in 5 years but 63.5% got disease relapse in 4 years. Three of them underwent salvage RIC allotransplant and in disease free. *Conclusions.* Mantle cell lymphoma is easy to remission after chemotherapy but easy to relapse soon as well. The long-term follow up survival curve does not see the plateau which indicated difficult to cure whatever regular or more intensive chemotherapy as the initial treatment. For relapsed patients, we could see the promising finding of durable survival in these patients (66.7% in 5 years) but need more longer follow up and more patients *Results.* We plan to perform clinical trial of RIC allotransplant as the frontline treatment for CR1 patients.

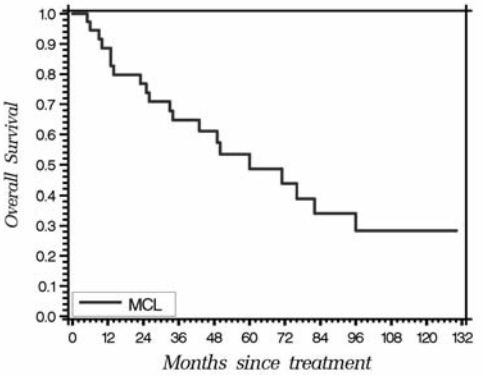

Figure. 8-year survival of Mantle cell lymphoma patients.

1608

TREATMENT OF SPLENIC MARGINAL ZONE NON HODGKIN LYMPHOMA(SMZL)

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The Splenic marginal zone lymphoma (SMZL) is a B-cell low grade neoplasm which has been recognized as separate entity with dinstictive clinical, immunophenotypic and histological features. *Aim.* To define the clinical characteristics and the therapeutic outcomes in SMZL. *Patients and methods.* Fifteen patients with SMZL were studied in our department. Diagnosis required splenic involvement, with or without bone marrow infiltration and the absence of excessive lymphadenopathy or evidence of extranodal (MALT) lymphoma. Patients' characteristics are presented in Table 1. Immunophenotypic features: Follicular or difuse infiltration with splenic sinuses involvement, extended from splenic marginal zone to red pulp, from small or medium sized centrocyte like or monocytoid B-cells, characterized by the immunophenotypic features of $CD20^+$, $CD79a^+$, $CD45^+$, IgM^+, $CD5^-$, $CD10^-$,$CD23^-$,Cyclin $D1^-$, and Bcl 6^-. Treatment included watch and wait, splenectomy, chemotherapy, and Rituximab as monotherapy in dose 375 mgr/m^2 weekly, for 4 weeks as induction therapy, and then one dose every 2 months for 4 doses totally as maintenance therapy. Six patients (40%) were treated with Rituximab monotherapy, five patients (33%) with splenectomy(four of them due to progressive disease received plus Rituximab monotherapy), three patients (20%) were treated with CVP like regimen or R-CHOP/with Caelyx because of extensive disease, and one patient (7%) was under observation ,watch and wait. *Results.* Overall response rate was 86%. Eight patients (53%) achieved complete response (CR) and three patients (20%) partial response (PR). In addition two patients are still on therapy, while a third patient had refractory disease to the CVP like regimen and presented allergic reactions after Rituximab administration. Median follow-up time for the entire serie was 36m(7-276). Only one patient who was under observation died during the follow-up period from other cause. *Summary/Conclusions.* The SMZL is characterized from moderate clinical course and long survival. The treatment is based mainly in splenectomy, while enough other therapeutic options have been applied. There are important evidence that Rituximab monotherapy may substitute splenectomy as first line treatment. This approach is particularly appealing in older patients, with other comorbidities and in cases of coexisting autoimmune events. The high response rate, the long duration of remission, the simple way of administration and the little adverse effects constitute

important advantages in favor of immunotherapy. The role of chemotherapy is discussing. The combination (immuno. and chemotherapy) appears effective but has not been compared to immunotherapy monotherapy. Splenectomy can be used in case of refractory disease to the Rituximab.

Table 1.

Characteristics	Number of patients	%	Characteristics	Number of patients	%
Age	76 yrs (55-82)		Peripheral lymphadenopathy	1/15	7
Hb<12gr/dl	6/15	40	Bonemarrow involvement	15/15	100
PLT<15x10⁴/mm³ PLT<5x10⁴/mm³	10/15	67	M-component	1/15	7
Neutropenia <1000/mm³	0/15	0	HCV-serology HBV-serology	0/15 0/15	0 0
Absolute lymphocytosis	9/15	60	Stage III-IV	15/15	100
B- symptoms	1/15	7	Extranodal sites	3/15	20
LDH> normal	5/15	33	Sex(M:F)	9/6	

1609

BIODISTRIBUTION AND RADIATION DOSIMETRY OF 90Y-IBRITUMOMAB-TIUXETAN USING 89ZR-IBRITUMOMAB-TIUXETAN AND IMMUNO- PET IN PATIENTS WITH B-CELL NHL

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Background. 89Zr-ibritumomab-tiuxetan-PET can be used to monitor biodistribution of 90Y-ibritumomab-tiuxetan (Zevalin®) as shown in mice. The aim of this study was to assess biodistribution and radiation dosimetry of Zevalin in humans using PET to evaluate whether co-injection of a therapeutic dose of Zevalin influences the biodistribution of 89Zr-ibritumomab-tiuxetan. If not, such combined administration may facilitate the prediction of the actual dose delivered to organs and tumor. *Methods.* Six patients with B cell NHL relapsing after anti-CD20 containing immuno-chemotherapy and scheduled for autologous stem cell transplantation underwent PET scans at 1, 72 and 144 h after injection of 50 MBq 89Zr-ibritumomab-tiuxetan and again after co-injection of 14.8 MBq/kg Zevalin. Volumes of interest (VOIs) were drawn over liver, kidneys, lungs, spleen and tumours. Zevalin organ absorbed doses were calculated using a special computer program (Olinda). Red marrow dosimetry was based on blood samples, taken at different time points. Tumor absorbed doses were calculated using an exponential fit. Agreement between uptake prior to and during therapy was assessed using intraclass correlation coefficients (ICC). *Results.* Highest 90Y absorbed dose was seen in the liver (4.4±2.5 mGy/MBq), followed by spleen, kidneys and lungs. No liver function abnormalities have been observed. There is significant uptake in lymphoma tissue, despite extensive pre-treatment with anti-CD20 (Figure 1).

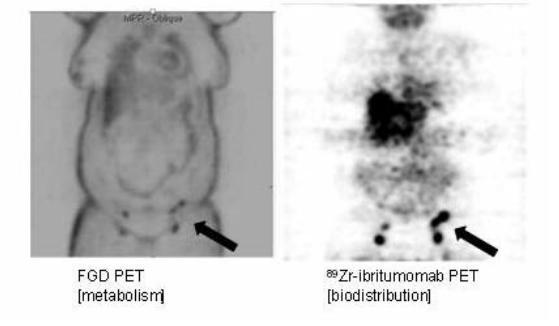

Figure 1.

Red marrow dose was 0.7±0.1 mGy/MBq. Effective dose was 0.7±0.2 mSv/MBq. Tumor doses ranged from 9 to 29 mGy/MBq. Pre-therapy and therapy uptake in source organs showed a high agreement (ICC = 0.97, mean difference 5±15%). Agreement between predicted 90Y effective doses was high as well (ICC = 0.91). No significant difference between pre-therapy and therapy tumor doses was found, but their correlation was lower (ICC 0.75, mean difference 5±33%). *Conclusion.* Biodistribution of 89Zr-ibritumomab-tiuxetan is not influenced by simultaneous therapy with Zevalin. Absorbed doses to kidney and spleen were significantly higher and lower, respectively, than those previously estimated using 111In-ibritumomab-tiuxetan. Combined administration of immunotracer and immunotherapy is feasible and facilitates actual biodistribution and dosimetry-calculations.

1610

PERIPHERICAL T CELL LYMPHOMA: DIAGNOSIS, TREATMENT AND SURVIVALL (PORTUGUESE ONCOLOGICAL CENTER'S EXPERIENCE)

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Background. Peripherical T cell non-Hodgkin Lymphoma (PTCL-NOS) is uncommon and has adverse clinical prognosis. They represent 23% of the T cell lymphomas treated at our Institution. *Aims.* Characterization, determination of the prognostic factors, treatment response and overall survival of the patients diagnosed with PTCL-NOS at Instituto Português de Oncologia do Porto, Portugal. *Methods.* We gathered a series of cases, based on retrospective revision of clinical data of patients diagnosed with PTCL-NOS, at our Institution, between January 1998 and July 2009. Statistical analysis was made on xlstat2009. *Results.* 25 patients were diagnosed (64%male, median 56 year old). Seventeen patients (68%) presented B symptoms, 10 (40%) were at III/IV Ann Arbor stage, 8 (32%) had IPI≥3. Fourteen patients (56%) presented extranodular involvement, 4 (16%) had bone marrow involvement, 9 (36%) patients had cutaneous involvement, 2 (8%) had CNS involvement, 2 (8%) had enterical involvement, 2 (8%) had pulmonary involvement and 1 (4%) patient had spleen involvement. Three patients had leukemic presentation. Median follow up was 30 months. Initial treatment was CHOP in 20 cases (80%), followed by radiotherapy in 6 cases (24%). There were 10 (42%) complete responses (CR), 10 (42%) progressions and 4 (16%) partial responses (PR). Most patients (6/10) that had progression had no adicional chemotherapy treatement, and had supportive treatment. In respect of the other patients that had progression, 2 of them received chemotherapy with alentuzumab+ ICE, one was treated with H-CVAD and another received ESHAP, as second line treatment. Only one had CR with alentuzumab+ICE and went consolidation with autologous bone marrow transplantation. All other patients died with disease (9/10). Two patients with PR are with active inaugural treatment (CHOP), while the two remaining patients with PR obtained CR with second line treatment (ESHAP and CHOP, respectively). Six patients (24%) initially with CR had relapse disease, 4 of them were treated with ESHAP and one with Alentuzumab+ICE. There were 5 (83%) CR, one of these patients underwent autologous bone marrow transplantation. There was also a progression, treated with alentuzumab+fludarabine, with CR. There were 11 deaths (44%) related with disease events, and 14 are still in follow up. Median overall survival is 71 months (stand. dv. 0,67months). Median overall survival, according IPI stage, is 55 months to IPI≤2, 6,5 months to IPI≥3, which is statistically different (wilcoxon 0,014).

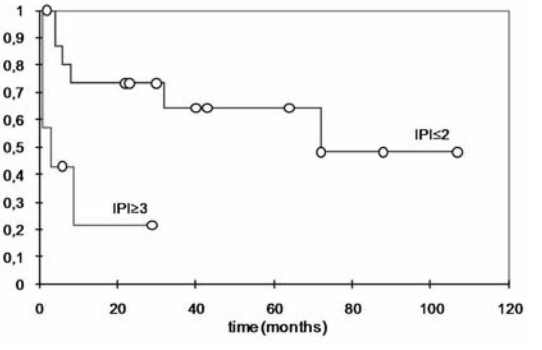

Figure. Overall survival PTCL.

Conclusion. PTCL-NOS is a disease with poor chemotherapy response, when compared with B cell Lymphomas. In 10 chemo-resistant patients to first line treatment, only one achieved CR. After CR in first line treatment, every relapse achieved CR, after 2nd or 3rd line treatment. IPI stratification could identify patients with worst prognosis. Intensified chemotherapy should be offered to these patients (alentuzumab or bone marrow transplantation).

1611

SECONDARY OSTEOPOROSIS AND SKELETAL RELATED EVENT (SRE) IN NON-HODGKIN'S LYMPHOMA TREATED WITH HIGH DOSE GLUCOCORTICOIDS

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Objectives. Treatment with glucocorticoids is associated with bone loss is an increased risk of fracture. This study was performed to determine risk of osteoporosis and skeletal related event associated with high dose steroid among non-Hodgkin's lymphoma (NHL) patients. *Methods.* Data were from retrospective medical database. From March 2004 to March 2008, newly diagnosed Non Hodgkin's lymphoma patients treated with high dose glucocorticoids containing chemotherapy were examined bone mineral densitometry (BMD) studies before and end of chemotherapy courses. Skeletal related event were evaluated during all course of treatment. *Results.* The prevalence of Skeletal related event defined as BMD -2.5 or less and 0.5 decline after treatment and development fracture is about 70%. Clinically meaningful and statistically significant factor associated with skeletal related event were age older than 60yrs, baseline decline of BMD, 0.5 or more loss of femur and spine BMD after treatment. In multivariable analysis, the age older than 60yrs and 0.5 or more loss of spine BMD are statistically significant risk factor. *Conclusion.* Risk of osteoporosis and Skeletal related event is relatively high in NHL treated with high dose glucocorticoids containing chemotherapy. Preventive support and comprehensive evaluation for osteoporosis is warranted especially in elderly and patients who have history of previous bone loss.

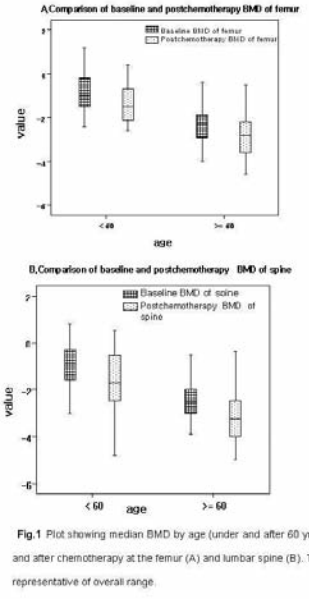

Fig.1 Plot showing median BMD by age (under and after 60 yrs) before and after chemotherapy at the femur (A) and lumbar spine (B). The bar is representative of overall range.

Figure.

1612

RADIOTHERAPY FOR THE INICIAL TREATMENT OF PRIMARY CENTRAL NERVOUS SYSTEM LYMPHOMA

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Background. Primary Central Nervous System Lymphoma (PCNSL) is a rare disease that accounts for less than 1 % of all non Hodgkin Lymphoma and about 3 % of primary CNS tumors. Until 1990 PCNSL was treated with radiotherapy (RT) alone. However, disease recurrence was high despite good initial responses. Chemotherapy based on high dose methotrexate (MTX) has been shown to improve survival compared to RT alone. However the optimal approach is still unclear. The role of RT in initial treatment regimen is not defined and the combined therapy can be associated with long term neurotoxicity (NT). Since 2006, trying to minimize NT we are treating patients without RT if they are more than 50 years old. Our aim was to compare progression free survival in patients treated with or without RT in initial treatment schedule. *Patients and Methods.* Twenty six immunocompetent patients with PSNCL were considered for high dose MTX in our institution between 1998 and 2009. After excluding systemic disease, diagnosis was done by biopsy of the lesion in all but one patient (cerebrospinal fluid (CSF) positive). Until 2006 we used high dose MTX according to Memorial Sloan-Kettering Cancer Center (MSKCC) 1990 protocol (Table 1). After that we started treating patients according to the 2002 MSKCC protocol (Table 2). *Results.* Twenty six patients initiated the protocol. Only 17 have completed the treatment (9 patients before 2006 and 8 patients after 2006) because infectious complications (n=5) and disease progression (n=3). Mean age was 61 years (range 35 to 84 years). All but one achieved complete remission (CR) at the end of the treatment. In the first group (before 2006) we lost 1 patient to follow up. The remaining 7 patients are in CR at 30 months follow up and 4 of them are still in CR after 60 months follow up. In the second group (after 2006) five patients had more than 50 years at diagnosis so RT was deferred. Four of them relapsed (18, 19, 22 and 32 months after diagnosis). Only one is in CR (44 months). Two patients with less than 50 years old were treated after 2006 (CR at 22 and 30 months follow up). None of the patients treated after 2006 had positive CSF. *Conclusions.* In the group of patients who did not receive RT in the initial treatment, progression free survival is shortened. We also point out that none of this patient received intrathecal MTX. Because patients that relapse end up including RT in their treatment the impact of deferring RT in NT was not assessed. We are reconsidering the role of RT in the initial treatment of PCNSL patients.

Table 1. MSKCC 1990.

MTX (1 g/m^2) (2 cycles)
Intrathecal MTX (6 cycles)
↓
Whole-brain RT + boost to the tumor bed
↓
Cytarabine (3g/m^2) x 2 (2 cycles)

MTX: methotrexate
VCR: vincristine
PCB: procarbazine

Table 2. MSKCC 2002, adapted.

MTX (3,5 g/m^2) + VCR + PCB[1] (5 to 7 cycles)
Intrathecal MTX (if positive CSF)
↓
Whole-brain RT (for patients under 50 years old)
↓
Cytarabine (3g/m^2) x 2 (2 cycles)

[1] PCB only in cycle 1, 3, 5 and 7
MTX: methotrexate
VCR: vincristine
PCB: procarbazine

1613

CENTRAL NERVOUS SYSTEM RECURRENCE IN AGGRESSIVE B-CELL LYMPHOMA: A SINGLE INSTITUTION EXPERIENCE

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Introduction. Central nervous system involvement is uncommon (nearly 5%), but serious and usually fatal complication of aggressive lym-

phoma. This single centre retrosprctive study investigated the clinicopathologic characteristic and outcome of patients with relapse in the central nervous system. *Methods.* We identified 15 patients (8 female and 7 male) with secondary central nervous system lymphoma (SCNSL), treated at our center from 2004 to 2009. The median age was 47 years (range 21-67); 5 patients had initial diagnosis mediastinal large B-cell lymphoma (PMLBCL) and 10 patients (pts) - diffuse large B-cell lymphoma (DLBCL). In addition to 5 pts with PMLBCL, 8 pts DLBCL presented with a extranodal disease (testis, breast, stomach, tonsil, bone). According to the age-adjusted International Prognostic Index score (aaIPI), 11 pts had IPI=2-3. Patients were previously treated with CHOP or MACOP-B regimen, 8 pts received Rituximab (375 mg\m^2 - N 4-6). *Results.* CNS relapse usually was defined as brain parenchymal involvement, only 1 pts has leptomeningial disease. The median time from diagnosis to CNS relapse was 6 months (range 1 to 40 months), 6 pts had SCNSL during or very shortly (1-3 months) after completion of chemotherapy. CNS was the only site of progression/relapse in 7 pts. Treatment of patients with CNS disease was quite heterogeneous, but usually systemic therapy incorporating HD-MTX (>1g/m^2), 7 pts received MCP (HD MTX on days 1, 15, 30, CCNU on day 1, procarbazine on days 1-10). The median progression-free survival was 7 months, overall survival - 31 months, 1 pts is 20 months on complete remission after MCP. *Conclusions.* Our data suggests that CNS relapse of aggressive lymphoma occurs early (median time to relapse 6 months). SCNSL was associated with extranodal involvement and increased aaIPI score. The combination of HD-MTX with CCNU and procarbazine is feasible and effective, however, given the small number of patients, the power of this analysis is limited.

1614

HIGH PREVALENCE OF HEPATITIS C (HCV) INFECTION AND FAVORABLE PROGNOSIS IN PRIMARY HEPATIC LYMPHOMA (PHL)

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Background. Primary Hepatic non-Hodgkin's lymphoma (PHL) is a rare disease, representing 0,4% of extranodal non-Hodgkin's lymphomas. To date, less than 150 cases have been published. We report nine patients with PHL diagnosed in 1995 and 2009 at our center, with a study of the viral status and the result of cytotoxic treatment. *Results.* Nine patients with PHL were identified. The disease occurred in middle-aged men (median age: 58 years). The main presenting complaint was right upper quadrant abdominal pain (4/9 patients). Tumour markers (α-fetoprotein and CEA) were normal in six patients tested. Liver scans demonstrated either a solitary nodule or multiple lesions. Pathologic examination revealed diffuse large B cell lymphoma in 6 patients, two case of centrocytic lymphoma and one case of T cell lymphoma. Six patients were HCV-positive. Seven patients received chemotherapy (6CHOP,1 R-CHOP), one received chemotherapy (R-FN), while a patient with a single focal lesion received surgical treatment. The complete remission rate was 100% (9/9); one patient, who had HCV-related cirrhosis, died of hepato-renal syndrome in complete remission from lymphoma, and another patient died of acute myeloid leukemia. *Conclusions.* The outcome of patients with PHL who are treated with combination chemotherapy seems excellent. The frequent association of PHL with HCV infection suggests a possible role of this virus in lymphomagenesis. HCV- infection does not appear to influence the outcome of therapy.

1615

COMPLETE REMISSION INDUCED BY FOTEMUSTINE AS SALVAGE TREATMENT IN RELAPSED PRIMARY CENTRAL NERVOUS SYSTEM LYMPHOMA: A CASE REPORT

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Background. Despite recent therapeutic advances, primary central nervous system lymphoma (PCNSL) shows the worst prognosis among all Non-Hodgkin lymphomas (NHL), with a 5-years survival rate ranging from 4-40%. Front-line therapy for patients with PCNSL is a combined chemo-radiation regimen, and more recently high-dose chemotherapy with autologous stem-cell support (ASCT) as consolidation therapy. This therapeutic approach has improved Complete Response (CR) rate and median survival, but the prognosis of PCNSL remains poor with high rate of fatal relapse. There is no effective salvage therapy for patients who fail or relapse after front-line treatment. Fotemustine is a third generation nitrosourea, with elevated lipophilic properties, which contributes to facilitate its passage through the blood-brain barrier and into malignant cells; the drug has been approved in the treatment of malignant metastatic melanoma and primary brain tumors. Recently the drug has been used in a novel fotemustine-based high dose conditioning regimen in lymphoma patients. We report here the case of a patient with relapsing PCNSL after front-line and consolidation therapy, who received fotemustine as salvage therapy. A 33-year-old patient was admitted to the hospital because of signs of intracranial hypertension. Computerized tomography scan (CT) and magnetic resonance (MR) revealed a cerebral mass localized in the median deep region of the brain. Immunoistochemical analysis of the tissue (stereotactic biopsy) diagnosed a diffuse B large cell PCNSL. Front line therapy consisted of two courses of high dose Methotrexate and three courses of L-VAMP (Vincristine, Cytosine Arabinoside, Dexamethasone) combined with inthratecal lyposomal cytarabine and whole brain radiation therapy at the total dose of 40 Gy followed by a boost of 10 Gy. At the end of front-line program CR was documented by negative cerebral positron emission tomography (PET). After three months a consolidation treatment with autologous stem cell support has been carried out. Conditioning regimen consisted of FEAM (Fotemustine 150 mg/m^2 on days -8 and -7; Etoposide 200 mg/m^2 on days -6, -5, -4 and -3; Cytarabine 400 mg/m^2 on days -6, -5, -4 and -3; Melphalan 140 mg/m^2 on day -2). After a follow-up of 16 months, a progressive hearing loss and ataxia appeared; CT scan and MR showed a cerebellar relapse. Fotemustine was administered every 15 days at the dose of 100 mg/m^2, as salvage therapy. Patient achieved a second CR after eight courses of fotemustine. Fotemustine was well tolerated with a main haematological toxicity consisting of grade II thrombocytophenia. No extra-hematological toxicities were recorded. After a follow up of six months patient is still in CR confirmed also by PET. *Conclusion.* At our knowledge this represent the first report of CR achieved with fotemustine as single agent in patients with PCNSL relapsing after high-dose therapy and stem-cell support. Is noteworthy that CR in this patient has been achieved despite previous exposure to fotemustine, suggesting that drug does not induce resistance. However more patients have to be treated before to define the exact role of this agent in the treatment of relapsing PSNCL.

1616

INFILTRATION OF ORBIT IN ADVANCED STAGE NON-HODGKIN LYMPHOMAS

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Involvement of extralympatic tissues and organs is of significance in course and managment of non-Hodgkin lymphoma. Our goal was to determine in a retrospective analysis the frequency of orbital involvement in advanced non-Hodgkin lymphomas and the influence of such affection on the short- and long-term efficacy of a treatment used. During 2004-2008 period 411 patients with advanced stage (CS III-IV) non-Hodgkin lymphoma were hospitalized in Internal Medicine and Chemotherapy Clinic. During initial diagnostics 17 of those patients (4.1%) were recognized with exophthalmus: 11 with uni- and 6 with bilateral.A lymphomatic infiltration of orbital cavity tissues was present in 12 patients with exophthalmus. The group with orbital infiltration consisted of 8 females and 4 males aged 48 to 79 years (mean - 67,4 years). DLBCL histological subtype occurred in 6 patients, SLL in 3, MALT in 2, and FL in 1 patient. After verification by biopsy an exophthalmus was found not to be caused by lymphomatic infiltration in 5 patients. Instead it was associated with superior caval vein syndrome in 2 patients and with hyperthyroidism in another 2. The underlying pathomechanism could have been not revealed in one remaining patient. Except for histological and ophthalmological examination, methods used for exophthalmus evaluation included ultrasonography of the orbit, CT/NMR, and since year 2006 also PET (in indolent lymphoma group avidity occurred in 2 out of 4 patients who had undergone the scan before the treatment). Because of advanced stage of the disease patients had been qualified to undergo chemotherapy: DLBCL according to R-CHOP scheme - 6-8 cycles, SLL according to COP or R-

CVP schemes - 6 cycles, MALT according to COP scheme - 4-6 cycles, FL according to R-CVP scheme - 8 cycles. Regarding orbital cavity pathologies, total postchemoterapeutic regression was achieved in 10 out of 12 patients, who therefore received no further treatment in that area. As chemotherapy failed to affect orbital lymphomatic infiltration in 2 remaining patients they were qualified for radiotherapy. Treatment *Results.* DLBCL = 3×CR + 2×PR + 1×PD->death; SLL = 1×CR + 2×PR; MALT = 1×CR + 1×PR; FL = 1×CR. Follow up lasted for 18-55 months (median 34 months). Relapse took place in 1 patient with MALT subtype after 27 months, whereas progression took place in 1 patient with DLBCL after 12 months, and one patient with SLL subtype after 21 months. No recurrence of orbital cavity infiltration was observed in patients who undergone successful chemotherapy (10 of 12) including those with relapse or progression of lymphoma itself. *Conclusions.* Infiltration of orbital cavity in advanced stage non-Hodgkin lymphomas does not deteriorate the prognosis. Standard chemotherapy is effective form of treatment ensuring remission of orbital infiltration in vast majority (10/12=83%) of cases. Current observation (median of 34 months) indicates this effect to be stable also in patients who experienced either progression or recurrence of lymphoma itself.

1617

SEVERE CASE OF HEPATITIS C INFECTION AT A FOLLICULAR NON-HODGKIN LYMPHOMA PATIENT UNDER RITUXIMAB MAINTENANCE THERAPY

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Background. We present this case due to the possibility of contacting a serious viral infection or activating a previous one of patients undergoing monoclonal antibody treatment for non-hodgkin lymphomas. *Aims.* The aim of presenting this case is to draw the attention of physicians to a very serious viral complication in lymphoma patients treated with rituximab maintenance. *Methods.* The methods of investigation were: paraclinical, hematologycal (biochemistry, tumor marker measured by ELISA, cantitative PCR) imagistical (abdominal ultrasound and CT-scan). *Results.* We present the case of a 39 years old patient with B cell follicular lymphoma diagnosed in 2007 with abdominal and retroperitoneal localization. Initially the histological confirmation being done from abdominal adenopathies. She underwent 8 courses of R-CHOP with very good evolution. Her treatment was continued with rituximab maintenance. 1 $^1/_2$ years after starting maintenance therapy she developed fatigue and a slightly elevated bilirubin levels (total bilirubin 2.05 mg/dL). Initially we considered relapse of the lymphoma but the CT-scan didn't confirmed the presence of adenopathies in the abdomen and retroperitoneum. Because of the presence of an ovarian chist we suspected ovarian carcinoma, the level of CA-125 being very high 1282 U/L. The gynecological exam and biopsy didn't confirmed this. The paraclinical examination confirmed a hepatic cytolysis with elevated transaminase: sGOT 154 U/L, sGTP 229U/L, GGT 116 U/L and the level of alkalic phosphatase being 105 U/L. The HBs antigen was absent and the tests were negative for HCV antibodies. The total bilirubin was 2.59 mg/dL. The gastroenterologist still suspected the relapse of lymphoma but we decided not to continue the monoclonal antibody treatment and to observe the patient for a possible viral infection. In 3 weeks the patient was addmited to our clinic in a very bad clinical condition with extreme fatigue, jaundice, generalizated oedema and a big quantity of ascites. The paraclinical examination showed the following: sGOT 393 U/L, sGPT 681 U/L, GGT 779 U/L and highly elevated levels of total bilirubin 24 mg/dL, level of total proteins 2.1 g/L. The repeated viral tests confirmed the presence of virus C and the viral replication by repeated PCR showed very high levels of HCV ARN: 215.291.400 UL/mL, the sensibility of the method being ≥15UL/mL). The treatment she received was interferon alpha 3×3.000.000 Ui/week, human albumin, acid ursodeoxycholic. In 3 weeks the evolution become slightly favorable with lowering level of bilirubin (BiT 7.67 mg/dL, BiD 7.45 mg/dL) and the slow disappearance of the ascites from the abdominal cavity. *Conclusions.* We consider this a case of severe hepatitis C viral infection at a patient with compromised immunity due to prior chemotherapy and monoclonal antibody therapy. We think it is necessary to follow very closely the patients with monoclonal antibody therapy for viral infection and also to take into consideration the possibility of false pozitive tumor marker levels in the case of concomitent viral infections.

1618

INDEX FOR SERUM GLOBULIN COMPENSATION IN DIFFUSE LARGE B CELL NON HODGIN LYMPHOMA

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Background. Low level of serum albumin have been used as prognostic factor for many malignant and other chronic diseases. Usually the decrease may be partly compensated by globular proteins but in some patients the failure of globulins to compensate may reflect advanced disease. *Aim.* We examined the prognostic value of globulin compensation index in patients with diffuse large B-cell non Hodgkin lymphoma (DLBCL). *Methods.* In Clinical Hospital Center of Rijeka, Croatia, 108 patients with DLBCL were analysed according the globulin compensation index (GCI) and other clinical and laboratory parameters. The GCI was determined using mathematical formula as described previously (F S Al-Joudy, Singapore Med J 2005;46(12):710). According to literature patients were classified into three categories: 1.negative GCI and negative compensation; 2.GCI of 0 to less than 1.0 with partial compensation and 3. GCI equal or greater than 1.0 with full compensation. *Results.* Negative GCI in patients with DLBCL showed a significant association with stage III/IV, > or =2 extranodal involvements and risk of high international prognostic index (P<0.05). The complete response (CR) rate (85.9%) in the full compensation (elevated GCI) was higher than in the negative or partial compensation (21.5%). The time to progression and overall survival were shorter in the group with negative GCI (P<0.05). *Summary/Conclusions.* GCI might be useful marker to indicate the extent of lymphoma involvement and prognosis in DLBCL patients.

1619

CHOP CHEMOTHERAPY FOLLOWED BY 90YT IBRITUMOMAB TIUXETAN (ZEVALIN) IN PATIENTS WITH UNTREATED DIFFUSE LARGE B CELL LYMPHOMA

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Background. Radioimmunotherapy represents a significant advance over unlabeled immunotherapy for the treatment of patients with B-cell non-Hodgkin's lymphoma. The response rates tend to be higher with patients who treated wtih fewer prior therapies. *Objective.* To investigate efficacy and safety of a treatment approach combining introduction chemotherapy with cyclophosphamide, doxorubicin, vincristine and prednisolone (CHOP) followed by a course of 90Yt ibritumomab tiuxetan in patients with untreated diffuse large B cell lymphoma (DLBCL) stage II, III, or IV. *Methods.* This is a prospective, single-arm, open-label phase II trial. Thirteen patients with untreated DLBCL stage II-IV received an introduction of 6 cycles of CHOP chemotherapy followed 4-6 weeks later by 90Yt ibritumomab tiuxetan. Efficacy and tolerability were evaluated. *Results.* 13 patients were enrolled; 8 were men and 5 women. The age ranged from 19.8 to 71.4 years. Six were in stage II, 6 stage III and 1 stage IV. After 8 weeks of treatment, 12 patients were in complete response, whereas 1 patient had relapse of the disease. With a median follow up time of 13.9 months (range = 2.6, 24.6), the 2-year progression free survival was 76.9% (10/13) and 2-year overall survival was 92.3% (12/13). No patients were discontinued from the study due to drug intolerability. *Conclusion.* The study regimen has demonstrated good efficacy and tolerability in patients with DLBCL.

1620

IMPROVED SURVIVAL IN PATIENTS WITH HIV-ASSOCIATED BURKITT LYMPHOMA TREATED WITH INTENSIVE CHEMOTHERAPY AND HAART

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Background. The incidence of Burkitt Lymphoma (BL) is increased in HIV/AIDS at 200-1000 fold the HIV-negative population. Standard chemotherapy yields a median overall survival (OS) of only 6 months. Intensive chemotherapy such as the Magrath regimen (CODOX-M/IVAC +/- rituximab) yields long term OS in non-HIV BL of >85%. Prior to availability of HAART, outcomes for HIV+ pts with lymphoma

were poor because of opportunistic infections, sepsis and progressive lymphoma on chemotherapy. In the HAART era, survival may be equivalent to HIV-negative pts with standard chemotherapy. Rituximab is routinely added to the CODOX-M/IVAC regimen in non-HIV pts with BL. *Aims.* To determine the safety and efficacy of intensive chemotherapy with CODOX-M/IVAC +/- R in HIV+ pts with BL. *Methods.* We reviewed all pts with HIV-associated BL receiving the Magrath regimen to identify the frequency of toxicity, BL response to chemotherapy and survival. *Results.* 12 pts were identified. Median age at BL diagnosis (dx) was 45 (range 32-56) years and all 12 pts were male. BL stage was: I, n=2; II, n=1; III, n=3; IV, n=6; limited stage/low risk, n=1; advanced stage/high risk, n=11. ECOG performance status (n=8) was: 0-1, n=4; >2, n=4. HIV risk factor (n=10): sexual, n=9; injection drug use, n=1; a prior AIDS-defining illness (n=11) was present in 1. Median CD4 count and HIV viral load at BL dx were 380 cells/mL and <40 copies/mL (undetectable; n=11). Coinfections were: hepatitis B (n=10), n=2; hepatitis C (n=11), n=1. 11 pts received HAART with chemotherapy. Number of Magrath cycles (of 4 planned) were: 4, n=4; 3, n=1; and 1-2, n=6 (2 pts are on treatment). 8 pts received rituximab with chemotherapy; all 12 received CNS prophylaxis; and 11 received G-CSF. Prophylaxis was: PCP, n=12; HSV/VZV, n=6; fungal, n=2. Complications were: bacterial infection, 10 episodes in 5 pts; febrile neutropenia, n=4; late neutropenia, n=4; peripheral neuropathy, n=2; increased liver function tests, n=1 (in a hepatitis C+ pt); skin reaction, n=1; hallucinations, n=1, oral thrush, n=1. Grade 3-4 hematological toxicity occurred in all pts but dose reduction or delay was required in only 2. All pts with late neutropenia recovered counts fully with brief G-CSF support. At a median follow-up of 9.6 (1.4-47) months, 10 pts (83%) are alive. Of 8 pts who received HAART and rituximab with chemotherapy, all 8 are alive and in remission. There were 2 deaths, both from progressive BL, both in pts not receiving rituximab, 1 in a pt with CNS involvement at dx (CD4 140 cells/mL at BL dx, did not receive HAART); neither death was from treatment-related toxicity. *Conclusions.* Patients with HIV-associated BL have acceptable tolerance of the Magrath regimen. All 8 patients who received rituximab with chemotherapy are alive and in remission. There were no deaths in pts receiving rituximab and HAART with chemotherapy. The survival of HIV+ pts with Burkitt Lymphoma without CNS involvement receiving intensive chemotherapy appears superior to that achieved with standard chemotherapy and is similar to non-HIV-associated BL, provided HIV control is optimized.

1621

HELICOBACTER PYLORI AND PRIMARY GASTRIC MALT LYMPHOMA

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Background. Helicobacter pylori has been claimed to be an important etiological factor which raises the risk of mucosa-associated tissue lymphoid (MALT) lymphoma. However, some studies on gastric MALT lymphoma revealed a low rate of H. pylori infection suggesting that not all gastric lymphomas are related to H. pylori infection. *Aim.* was to verify the H. pylori infection frequency in a series of patients with primary gastric MALT lymphomas and to examine the relationship between H. pylori and the pathological features of those lymphomas. *Methods and results.* 14 cases of gastric lymphoma were analysed: 9 cases (64,28%) were low-grade MALT lymphomas and 5 cases (35,71%) were high-grade MALT lymphomas. Helicobacter pylori was found in 12 of 14 (85,71%) cases. Helicobacter pylori infection was significantly correlated with the grade and depth of invasion of MALT lymphoma since over 64% of superficial low-grade MALT lymphomas were positive for H. pylori compared with 35,71% of advanced high-grade MALT lymphomas. *Conclusion.* We confirmed the relationship between H. pylori infection and a subset of gastric MALT lymphoma. Our results also showed that not all low- and high-grade gastric MALT lymphomas are H. pylori-dependent. This suggests that H. pylori infection may play a promoter role in the development of MALT lymphoma. Because the groups were small in size, statistical significance was not achieved.

1622

AUTOLOGOUS STEM CELL TRANSPLANTATION IN ADULT PATIENTS WITH RELAPSED OR PRIMARY REFRACTORY PERIPHERAL T-CELL LYMPHOMA

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Background. Autologous stem cell transplantation (ASCT) is established as salvage therapy for chemosensitive relapsed or primary refractory aggressive lymphoma such as diffuse large B-cell lymphoma (DLBCL). But it is not clear whether ASCT improve the prognosis in the subset of patients with peripheral T-cell lymphoma (PTCL). *Aims.* The aim of this study was to evaluate the efficacy of ASCT in adult patients with chemosensitive relapsed or primary refractory PTCL. *Methods.* Between January 1996 and December 2008, 14 consecutive patients (median age 47.5 (22-62), male/female; 10/4) with chemosensitive relapsed or primary refractory PTCL who underwent ASCT were included. Patients with anaplastic lymphoma kinase (ALK) expressing anaplastic large cell lymphoma (ALCL) were excluded. The results of 14 patients with PTCL were compared with those of 32 consecutive patients (median age 55.5 (23-67), male/female; 24/8) with chemosensitive relapsed or primary refractory DLBCL who underwent ASCT during the same period. The χ^2-test was used for the binary variable comparison. The Mann-Whitney U test was used for the continuous variable comparison. Overall survival (OS) and event free survival (EFS) were estimated by the Kaplan-Meier method, and compared using the log-rank test. The Cox proportional hazards regression model was used for the multivariate analysis of prognostic factors. P<0.05 was considered to indicate a statistical significance. *Results.* Histological PTCL subtype included PTCL not otherwise specified (n=4), ALK-negative ALCL (n=6), angioimmunoblastic T-cell lymphoma (n=3), and subcutaneous panniculitis-like T-cell Lymphoma (n=1). No significant differences in age, sex, international prognostic index (IPI) at diagnosis, and conditioning regimen contained total-body irradiation or not were demonstrated in both groups. The proportion of patients in CR and PR at the time of ASCT was not significantly difference in both groups. The 5-year PFS rates for patients with PTCL and DLBCL were 29% and 37% respectively (P=0.62). The 5-year OS rates for patients with PTCL and DLBCL were 32% and 48% respectively (P=0.75). In patients with PTCL the 5-year PFS rates for patients in CR and PR at the time of ASCT were 67% and 18% respectively (P=0.11), and the 5-year OS rates were 100% and 18% respectively (P=0.04). Disease progression occurred in 82% of patients in PR at the time of ASCT within 1-year. Multivariate analysis of both OS and PFS in patients with PTCL showed an independent prognostic significance of only a CR status at the time of ASCT. *Conclusions.* The prognosis after ASCT in patients with chemosensitive relapsed or primary refractory PTCL and DLBCL were similar. Based on this result patients with relapsed or primary refractory PTCL should be treated with salvage therapy with the intent of ASCT for patients with chemosensitive disease. However, the prognosis in patients who did not achieve CR at the time of ASCT was dismal. To improve the outcome of these patients allogenic stem cell transplantation should be investigated.

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CNS INVOLVEMENT IN A CASE OF HUMAN T LYMPHOTROPIC VIRUS TYPE 1 (HTLV-1) ASSOCIATED LEUKEMIA/LYMPHOMA

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Background. Central nervous system involvement in adult T-cell leukemia/lymphoma is rare and has been demonstrated with with mass lesions on imaging. We want to present a 20 -year-old woman,known case of lymphoblastic lymphoma in complete remissin who admitted with seizure and comatose state and further evaluation revealed HTLV-1 antibodies in her serum and CSF.Serial brain imaging revealed CNS involvement. We started Dexamethasone, Zidovudine, interferon and Systemic chemotherapy including High dose of Methotrexate and then rain radiation and saw good clinical response. *Case Report.* A 20 y/o married female a case of lymphoblastic lymphoma admitted in ICU

with loss of consciousness and seizure. She was history of Non - Hodgkin lymphoma (lymphoblastic-type) one year ago and received chemotherapy in another center. At presentation time she had cervical lymphadenopathy, splenomegaly and bone marrow involvement. After 8 cycle of chemotherapy (CHOP)regimen she had achieved complete remission. Her family complained history of ataxia, diplopia and vertigo since 2 week PTA and 3 times seizure in the day of admission. Physical examination showed comatose state, nystagmus,bilateral extensor plantar reflexes.Laboratory findings are included hypercalcemia,normal CBC diff, BUN, Cr, Na, K, Liver function test.Brain CT scan with contrast was normal and cerebrospinal fluid(CSF) analysis revealed acellular CSF with clear appearance, 47 mg/dL glucose and 28 mg/dL protein. HTLV -I antibody(ELISA) was positive in blood and CSF. Brain CT was repeated after one week and revealed a 3x3cm mass in the left sided deep white matter near basal ganglia.Initially we started Dexamethasone, Pamidronate and Intratechal chemotherapy and antiviral therapy including Interferon 3,000,000 unit 3 times a week & Zidovudine 300 mg q12 h then we administered systemic chemotherapy included high dose methotrexate (3.5 gr MTX) with leucovorine rescuse. After several days her level of consciousness was rised slowly and she was be able to move her left sided upper and lower extremities. After ten day brain radiotherapy was done for her which leads to significant increase in level of consciousness ,and she was be able to open her eyes and obey the orders and speech but right sided hemiparesis was persisted. *Result.* In this case of lymphoblastic lymphoma who was admitted with seizure and comatose state,despite initially normal reported brain CT scan with contrast and acellular CSF we found HTLV-1 antibodies in Blood and CSF. We repeated brain CTscan after one week that showed a 3×3 Cm mass lesion in left sided deep white matter near basal ganglia. We started antiviral therapy with Interferon and Zidovudine and saw a good clinical response after administration of high dose of MTX with leucovorine rescues followed by brain radiotherapy. *Conclusion.* We recommend searching for HTLV-1 Ab in any case of T-cell lymphoblastic lymphoma and neurologic finding although in presence of acellular CSF with norma glucose and protein. We also recommend serial Brain imaging in the presence of positive neurologic findings in patient with adult T-cell leukemia lymphoma. Combination of antiviral therapy with systemic chemotherapy included high-dose MTX with leucovorine rescues and brain irradiation are recommended in patients with adul T-cell leukemia/lymphoma and CNS involvement which presents of mass lesion in brain imaging.

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COAGULATION DISORDERS IN ELDERLY PATIENTS WITH NON HODGKIN'S LYMPHOMA

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Acquired hemophilia is a rare coagulation disorder characterized by autoantibodies against circulating coagulation factor, frequently against factor VIII. Patients often have not history of bleeding disorder and present spontaneous hemorrhage, an isolated prolonged aPTT and PT and antibodies against coagulation factors. It has an incidence of 0,2-1 cases/million/year. This condition may be associated in 50% of cases with autoimmune disease, solid tumor, lymphoproliferative disorders and pregnancy. We describe our experience with two patients with indolent non-Hodgkin's lymphoma who showed isolated prolonged aPTT and PT. *Case one.* A 72-year-old man referred to our Institution because of recurrent epistaxis and abnormalities of coagulation tests PT INR 2.5, a PTT ratio 2.73. No previous personal or family history of bleeding disorders, or recent surgery and new drug intake, were reported. Spleen enlargement, with a large focal lesion, and pancytopenia (Leukocytes 2800/μL; Haemoglobin 9 g/dL; Platelets 82,000/μL)were observed. Laboratory test showed a reduction of Factor VIII activity, Factor II activity, Factor V activity, Factor VII activity, Factor IX activity, Factor activity X and Factor activity XI (FVIII:C 16%; FII: 44%; FV:8%; FVII: 11%; FIX: 10%; FX:30%; FXI:27%) and appearance of antibodies against many coagulation factors. The bone marrow showed a lymphoid infiltrate. Fluorodeoxyglucose F-18 positron emission tomography whole-body scan revealed abnormally high uptake in the spleen and in a slightly enlargement supraclavear lymphonode. Fine needle aspiration biopsy at this site, covered by 60 μg/Kg recombinant Factor VIIa (NovoSeven) injections, enabled the diagnosis of non-Hodgkin's lymphoma ($CD5^-$, $CD22^+$, $lamba^+$). The patient made six administration of chemotherapy with cyclophosfamide, vincristine, epirubicin and prednisolone (CEOP) and achieved complete remission. We observed that the end of treatment, coagulation parameters and factor activity returned normally. *Case two.* A 62 year-old female came to our observation for lymphoadenopathy, hepatoplenomegaly, anemia and lymphocitosis. The patient didn't present any personal or family bleeding disorders and didn't take drugs. Laboratory tests showed abnormalities of coagulation: PT INR 3.26, aPTT ratio 4.92. They also showed a reduction of factor VIII activity, Factor II activity, Factor VII activity, Factor IX activity, Factor activity X and Factor activity XI (FVIII:C: 2.3%; FII: 32%; FVII: 47%; FIX: 1%; FX:43%; FXI:1%) and appearance of antibodies against many coagulation factors. Fluorodeoxyglucose F-18 positron emission tomography whole-body scan revealed increased uptake at axillary and inguinal lymphonodes and at spleen. The bone marrow showed a lymphoid infiltrate and enabled the diagnosis of non-Hodgkin's lymphoma ($CD5^-$, $CD22^+$, $lamba^+$). The patient made two administration of chemotherapy with Rituximab, fludarabine. After second administration of chemotherapy, was observed a slow normalization of PT and aPTT. In both cases there was a correction of the PT and aPTT after the first cycle chemotherapy. In these patients the onset of an acquired coagulation disorder can be used as diagnostic and prognostic marker of derangement of the immunologic network due to an underlying, apparently indolent, lymphoproliferative disease.

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SINUS HISTIOCYTOSIS WITH MASSIVE LYMPHADENOPATHY ASSOCIATED TO DIFFUSE LARGE B CELL LYMPHOMA

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Background. Sinus Histiocytosis with Massive Lymphadenopathy (SHML) is a rare benign disorder characterized by painless lymphadenopathy. Cervical lymph nodes are most frecuently involved. Tipically, the lymph nodes are expanded because of a proliferation of histiocytes that sometimes show emperipolesis of lymphocytes. Extension below diaphragm is unusual, as is association with other lymphoproliferative disorders. We report a case of SHML associated to Diffuse Large B Cell Lymphoma. *Case report.* A 72 year old woman was studied because of elevated transaminases. Physical examination revealed an enlargement of a right inguinal lymph node. There were no skin eruptions. Mild anemia, elevated Beta-2-microglobulin and LDH were found. CT-Scan revealed enlargement of lateroaortic and inguinal lymph nodes in addition to multiple nodules on lungs and spleen. A PET confirmed lungs and spleen involvement, as well as axial bones infiltration. Right inguinal node biopsy led to diagnosis of SHML. After 4 months' Prednisone therapy a new CT-Scan was performed. It revealed mediastinic and axilar lymph nodes enlargement and an increase of size and number of lung and spleen nodules. A bone marrow biopsy showed no alterations. Clinical worsening prompted a treatment change to Clorambucil and Prednisone cycles, soon abandoned due to side effects. Two months later, the patient was admitted to our Hospital because of abdominal pain, weigth loss and fatigue. A new CT-Scan revealed progression of previous tumours as well as a new pelvic mass infiltrating bladder, rectum and iliac bone. Biopsy of this mass showed SHML and Diffuse Large B Cell Lymphoma. Of note, both hystologic patterns were present in the same biopsy specimen. Repeated bone marrow biopsy, found infiltration by Lymphoma. Final diagnosis was extended SHML and stage IV-B Diffuse Large B Cell Lymphoma. The patient started treatment with Cyclophosphamide, Doxorrubicine, Vincristine, Prednisone and Rituximab (R-CHOP). She is still receiving treatment. *Comments on this case.* SHML seldom extends below diaphragm; and rarely progresses to cause symptoms. A few cases of association of SHML and Diffuse Large Cell Lymphoma have been reported to date.

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DIFFUSE LARGE B - CELL LYMPHOMA IN THE COURSE OF COMMON VARIABLE IMMUNODEFICIENCY SUCCESFULY TREATED WITH IMMUNOCHEMOTHERAPY: A CASE REPORT

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Background. Common variable immunodeficiency (CVID) is the primary hypogammaglobulinemia of unknown aetiopathogenesis. The common denominator is a dysregulation of immune responses affecting T and B cells with both central and peripheral tolerance mechanisms being disturbed. It is characterized by low levels of IgG/IgA antibodies, recurrent respiratory tract infections, granuloma formation, and the predisposition to malignancies. Lymphoma is one of the most severe complications of CVID. *Aim.* We report a patient with diffuse large B cell lymphoma (DLBCL) developed 3 years after the setting of CVID diagnosis sucesfully treated with immunochemotherapy. *A case report.* A 55-year old female patient was admitted on Institute of hematology with a high fever, bilateral cervical lymphadenopathy, splenomegaly, and fingernail thickening due to fungal infection. In the past history of this patient CVID associated with lung and lymph node granulomatosis was diagnosed and substitutive intravenous immunoglobulin (IVIg) therapy was inititate at a dose of 0.5 g/kg monthly. Laboratory test results showed mild normocytic anemia, 109 g/L, WBC 10×10^9/L; with granulocytosis, 8.1×10^9/L, lymphopenia, 1.1×10^9/L; PLT 283×10^9/L. Analysis of biohumoral parameters showed increase in sedimentation rate 36 mm/h and fibrinogen 5.1 g/l. She had severe hypogammaglobulinemia, IgG was 2.55g/L (N: 6.78-12.60), with mild decrease in IgA (0.26g/L, N: 0.82-2.62). Flow cytometry analysis of peripheral blood lymphocytes revealed a significant decrease in $CD4^+CD45RA^+$T cell count and increase in $CD4^+CD45RO^+$ T cell count. The T cell population ($CD3^+$) was 90% among T lymphocytes, with apparent excess in T suppressor cell subset ($CD3^+CD8^+$) - 30% and decrease in NK T cell subset - 1.5%. The relation between T helper and T suppressor was 0.75 which is diminished comparing to normal. Population of B - lymphocytes was minor and polyclonal, and the relation between lymphocytes expressing kappa and lambda light chain was within the reference value (1.75, N: 1.10-1.80). Virusological markers for HIV, HBV, HCV and EBV infection were negative. Immunohistochemical analysis of lymph node sections revealed a diffuse large B cell lymphoma ($CD20^+$, $CD79\alpha^+$, $CD3^-$, $CD5^-$, $bcl2^-$, MUM1-/+, Ki 67 40%). Disease was staged as IIB S^+ CS. Immunochemotherapy R-CHOP-21 was initiated along with monthly IVIg therapy. After VI cy patient achieived complete remission of lymphoma with continuing substitution of hypogammaglobulinemia. *Conclusion.* Relative risk of malignant lymphoma development in patients with CVID resembles the same risk in patients with HIV/AIDS patients. The specific phenotypic T cell changes in CVID patients correspond to those in viral infection and correlate with the B cell immaturity. That enhances importance of good viral infection prevention in CVID patients. The resulting immune dysregulation may lead to self-reactivity with developing malignancy. Combined immunochemotherapy in this condition induced lymphoma remission without any negative influence on immune deficienty.

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'SEVERE AUTO-IMUNE THROMBOCYTOPENIA AND THYROIDITIS AS PRESENTATION OF NON-HODGKIN B-CELL LYMPHOMA - A CASE STUDY'

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Background. B cell neoplasms are clonal tumours of immature and mature B cells, which comprise over 90% of lymphoid neoplasms worldwide. Mature B-cell neoplasias are often associated with immune processes, which are also, either immunodeficiency or autoimmune diseases, major known risk factors for the development of these neoplasias. Frequently, morphology and immunophenotype are sufficient for the diagnosis of lymphoid neoplasms. However, no one antigenic marker is specific for any neoplasm, and a combination of morphologic features and a panel of antigenic markers are necessary for correct diagnosis. *Aims.* Present a case study of a patient with auto-immune thyroiditis and severe auto-immune thrombocytopenia preceding Non-Hodgkin B-Cell Lymphoma (NHBCL). *Methods.* The patient was clinically evaluated and submitted to some diagnostic tests: full blood count, biochemical tests, viral serology, anti-platelets, anti-thyroid and anti-cardiolipin antibodies, ANCA's, ENA's, abdominal ultrasound and computorized tomography, bone marrow aspiration, immunophenotyping (flow citometry), kariotype, FISH technique, spleen histology. The patient was treated with corticotherapy, immunoglobulin and R-CVP (one cycle), followed by cyclosporin and R-CHOP (six cycles), and later on did splenectomy. *Results.* Personal antecedents: thyroid node in 2006, medicated with levothyroxin for six months and asthma. Clinical features: haemorrhagic discrasia of oral mucosa, splenomegaly, no lymphadenopathy. Laboratory findings: platelets severe and persistently decreased (1 to $5,7\times10^9$/L), unresponsive to therapy until splenectomy. No anaemia, white blood cells including lymphocytes counts normal, with heterogeneous morphology, without villous lymphocytes. Biochemical tests: elevation of IgG, slightly decreased IgM, elevated free T3 and T4 and decreased TSH after first cycle of R-CHOP, normalized post-therapy. Proteinogram normal. Viral serology: negative, except for EBV IgG. Anti-platelets antibodies: positive, that soon responded to corticotherapy. Anti-thyroid antibodies: anti-tiroglobulin antibodies negative, with TRAB positive, becoming negative with corticotherapy and immunosupression. Anti-cardiolipin antibodies: IgM slightly increased. ANCA's and ENA's: negative. Abdominal computorized tomography: hepatosplenomegaly without adenomegaly. Bone marrow (BM) aspirate: 50% infiltration of small lymphoid cells, some with less dense chromatin and irregular nucleous contour. BM immunophenotyping (IF): 9% of lymphoid cells CD5, CD10, CD103 negative, CD23, FMC7, CD79b, CD20, CD25 positive and lambda weak. Peripheral blood IF: 18% of lymphoid cells, with same phenotype. Genetics: 46, XY(20). Fish technique: negative for t(14:18) and t(11;14). After two cycles of chemotherapy BM aspirate and biopsy showed no infiltration of lymphoid cells and megacariocytes numbers and morphology of immune thrombocytopenia. Spleen histology: no infiltration. *Summary/Conclusions.* This is an interesting example of an autoimmune process (thyroiditis and thrombocytopenia) that preceded the lymphoma diagnosis, supporting some published studies. The patient presented a very good response to chemotherapy and all the immune factors were rapidly controlled with corticotherapy and immunosupression. However, despite antiplatelets antibodies being already negative, the patient maintained a severe thrombocytopenia, probably due to hypersplenism. Platelets counts normalized, only after splenectomy. Curiously, spleen histology (after therapy) did not revealed infiltration by lymphoid cells. Conjugated with the fact that there was no adenomegaly, the results of morphology, immunophenotyping, and cytogenetics, it was not possible to classify with certainty the sub-type of this splenic lymphoma (splenic marginal zone lymphoma/splenic diffuse red pulp small B-cell lymphoma).

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PRIMARY DUODENAL FOLLICULAR LYMPHOMA: REPORT OF TWO CASES

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Background. Gastrointestinal (GI) tract is the most frequent site of extranodal non-Hodgkin lymphoma (ENHL), and the stomach is predominantly involved. Instead, the duodenum is rarely affected by ENHL. High-grade lymphomas are the most common histological subtype affecting the GI tract excluding the stomach, where MALT lymphoma represents nearly half of all cases. Although nodal follicular lymphoma (FL) is the most common subtype of indolent lymphoma, extranodal FL represents only the 7% of GI lymphomas. Consequently, primary FL of the duodenum is rare and few cases are reported in the literature. The gold standard treatment of this disease is unknown. *Aims.* To retrospectively evaluate the outcome of two patients (pts) affected by primary duodenal FL treated with combination of rituximab and CVP polichemotherapy (R-CVP). *Methods.* Two pts, women aged 47 and 68 years, were admitted to our hospital with a diagnosis of duodenal NHL. In both cases histological reevaluation of biopsy specimens found a lymphoid infiltrate of the duodenal mucosa composed of predominantly CD20, CD10 and Bcl2 positive small-cleaved cells, consistent with FL. Total body CT scan and bone marrow (BM) biopsy showed no abnormalities. Both pts presented Bcl2-IgH rearrangement in BM. The stage of disease was evaluated as IE A according to Ann Arbor staging system and the FLIPI score as 0. Both pts received 4 courses of R-CVP. *Results.* After treatment completion, the pts received esoph-

agogastroduodenoscopy and BM biopsy. There was no detectable lymphoma in the duodenal biopsy specimens and no detectable Bcl2 rearrangement in BM. Both pts are alive and lymphoma-free so far, after 23 and 36 months respectively. *Conclusions.* Different treatment strategies such as radiotherapy (RT), chemotherapy (CHT) and rituximab, alone or in combination have been tried in early stage FL. Rituximab combined with CHT could improve overall survival for pts with advanced FL. Rituximab with or without CHT is also effective in primary duodenal FL. We believe that rituximab combined with CHT could be a very good option to achieve clinical and molecular response. We submitted our pts to only four courses of R-CVP because of their low tumor burden. Considering that median time to retreatment is longer than 30 months in advanced FL treated with R-CVP, we are unable to evaluate long-term significance of molecular response in our pts today because of short follow up. Nevertheless, we think that this result is very promising because pts who achieve a molecular response have a longer failure free survival than those who do not.

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THE FIRST EXPERIENCE IN THE TREATMENT OF DIFFUSE LARGE B-CELL LYMPHOMA WITH BONE MARROW (DLBCLWBM) INVOLVEMENT BY MODIFIED PROGRAM NHL-BFM-90 AND SEQUENTIAL HIGH-DOSE CHEMOTHERAPY WITH AUTO-SCT (MNHL-BFM-90$^+$ SHD$^+$ASCT)

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Background. DLBCLwBM has poor prognosis. In standard therapy (CHOP, R-CHOP) the risk of relapse is high. The 3-year OS is 38% on CHOP regimen. *Aim.* to evaluate efficacy and safety of the mNHL-BFM-90+SHD+ASCT for DLBCLwBM. *Methods.* five pts (all men, 23-47 years, middle 37) with DLBCLwBM were treated between Feb 2007 and Dec 2009. The patterns of bone marrow lymphoid infiltration were diffuse large-cell in 3 cases and nodular mixed-cell in 2 cases. Three pts had generalised lymphadenopathy, one-dorsal vertebra lesion, one- isolated tumor in caecum. In all cases bone marrow lymphoid infiltrates were evaluated by morphology, immunohistochemistry and molecular analyses (PCR). The NHL-BFM-90 program (block A and block C) was modified in the following way: doxorubicin 50mg\m^2 was added on the third day of block A, methotrexate 1g\m^2 i\v was added on the first day of block C. All pts received mNHL-BFM as first line therapy from 4 to 6 blocks continued by SHD. Following high-dose cyclophosphamide 3 pts achieved the target number of CD34$^+$ cells, 1 after high-dose etoposide. Four pts as conditioning regimen have received BEAM with ASCT. One failed stem-cell mobilization and was excluded. *Results.* All patients achieved complete remission at the beginning of SHD. One had relapse in 5 months after BEAM. He received salvage chemotherapy. All pts are alive. The mean follow-up is 23 month (range 15-30). Hematologic toxicity of grade 3 and 4 was observed, the severe infectious complications were not registered. *Conclusions.* mNHL-BFM-90$^+$ SHD$^+$ASCT is well tolerated and effective in pts with DLBCLwBM. Further study and longer follow-up are necessary.

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COEXISTING PLASMOCYTOSIS OF THE SKIN AND LOW GRADE B LYMPHOMA: REVIEW OF LITERATURE AND CASE REPORT

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Introduction. The plasmocytosis of the skin and systems (PCS) is rare not neoplastic reactive lymphoplasmocytic disorder that affecting mainly the far est, particularly the Japanese. Initially it was known as plasmocytosis skin, today is also called systemic for extracutaneous involvement. Clinically, its distinctive feature is polymorphous lesions (macules, papules, plaques, nodules) of various color of the skin. There may be superficial lymphadenopathy with interfollicular infiltrates of polyclonal plasma cells, anemia, hepato-splenomegaly and pulmonary, renal, retroperitoneal and breast involvement. *Case report.* A 62 years old men was observed by us for confluent bright red lesions, raised of his face appeared for several months. He had only a moderate itching. There was no superficial lymphadenopathy, anemia, weight loss or fever. CT scan and PET scan showed only one lymph node at the chest. Two biopsy specimens were taken from the cheek and the nose and histological examination revealed a normal epidermis, but dense interstitial and perivascular infiltrated of mature plasma cells (CD138$^+$) in the dermis; no clonal restriction for immunoglobulin light chains to immunohistochemical staining. Polymerase chain reaction revealed no evidence of a clonal gene rearrangement of immunoglobulin heavy chains. Serological tests for Borrelia burgdorferi and syphilis were negative. The blood tests pointed high lymphocytes cell count and erythrocyte sedimentation and a double monoclonal gammopathy IgM k with elevated urinary free kappa light chains. No Bence-Jones protein was detected. A bone marrow biopsy specimen revealed a moderately increased number of mature plasma cells, and slight lymphocytic B infiltration compatible with lymphocitic non Hodgkin's Lymphoma. Flow-cytometric analysis of the bone marrow aspirate showed pathological elements lymphoid B, CD 19$^+$, CD20$^+$, CD22$^+$, light chain restriction of type k compatible with chronic B disorder. No therapy was performed and the patient is only in "watch and wait". *Discussion.* First description of PCS in literature was in 1976 by Yashiro and are about one hundred the cases described in literature; it prefers the adult males. The histology showed a infiltrating pupils perished in the superficial and deep dermis consisting of mature plasma cells. The lymphoplasmocitic infiltration can remember a primary cutaneous B-cell lymphoma. Immunohistochemistry demonstrates the infiltrators with polyclonal light chain expression of kappa and lambda. At the bone marrow plasma cell population is usually normal. In most cases, the PCS has a benign course. The diagnosis of PCS is mainly based on exclusion and polyclonal plasma cell infiltration of the skin. The etiology is unknown and often is associated with POEMS syndrome and Castleman's disease. The IL6, elevated in the serum of this patients, has a role. Treatments consist in topical and or systemic corticosteroides, topical tacrolimus or pimecrolimus, PUVA therapy, cyclophosphamide, rituximab or local radiation. We must follow the disease for many years for possible malignant transformation. *Conclusion.* the case described is characteristic because it combines the presence of a plasmocytosis which is a skin condition that potentially evolve into malignant disease with a low-grade B lymphoma with minimal expression at the bone marrow and peripheral blood without clinical expression.

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EFFICACY AND SAFETY OF ORAL BEXAROTENE IN THE TREATMENT OF PRIMARY CUTANEOUS T-CELL LYMPHOMAS

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Background. Cutaneous T-cell lymphoma (CTCL) is a heterogeneous group of lymphoproliferative disorders, characterized by the infiltration of the epidermis by mature and activated malignant T-lymphocytes. Bexarotene, a highly selective retinoid X receptor (RXR) retinoid, is approved for the treatment of all stages of CTCL in patients refractory to at least one systemic therapy. CTCL is a rare disease and data on the use of bexarotene are limited. *Aims.* To summarize the clinical experience with oral bexarotene for patients with CTCL, with the aim of assessing efficacy and safety in the setting of a large Dutch university hospital. *Methods.* All patients commenced on bexarotene were identified from patient records at the departments of Hematology and Dermatology at Erasmus MC Rotterdam, the Netherlands. Diagnosis was obtained using skin histology, immunohistochemistry and T-cell receptor gene analysis. Patients were staged according to the WHO-EORTC classification. All patients were discussed in the Dutch National Workgroup for CTCL at time of diagnosis. Patients started with bexarotene 150 mg/m^2 daily titrating up to 300 mg/m^2 if tolerated. All patients received lipid lowering agents and thyroxin substitution before bexarotene start. *Results.* We studied 10 patients. Six had Mycosis Fungoides (MF), one a folliculotropic MF, one Sezary syndrome, one subcutaneous panniculitis-like T-cell lymphoma, and one unspecified primary cutaneous peripheral T-cell lymphoma. Five patients had early stage disease, the other five had advanced stage disease. Patient age at starting treatment ranged from 20 to 88 years with a mean age of 59 years. All patients received extensive local and/or UV therapy and/or systemic therapies (multichemotherapy, cyclosporine and prednisone) in the past before starting with bexarotene. During bexarotene therapy seven out of ten patients used additional systemic (prednisone n=1) or UV therapy (n=2), or local radiotherapy (n=1) and/or local steroids (n=6). Three patients used bexarotene monotherapy. Overall nine out of ten (9/10) patients responded during bexarotene therapy. One patient had a sustained complete response and eight patients had a

partial response. One patient had stable disease. The time needed for the first response to occur since bexarotene use varied from 0.2 months to 15 months, with a mean of 2.3 months. The duration of response varied from 2 months to more than 24 months. Out of the nine responders, three patients progressed while on therapy. These patients all had advanced stage CTCL. One patient died because of cardiopulmonary disease not related to bexarotene. Most frequently observed adverse side-effects experienced by our cohort were central hypothyroidism (n=6), hyperlipidemia (n=3) and hypertriglyceridemia (n=7). These side effects could be managed with thyroid replacement and lipid-lowering therapy. Other side effects were pruritis (n=1), hair loss (n=2), high liver enzymes (n=3) and leucopenia (n=1). *Conclusions.* Our data demonstrate that bexarotene produces durable responses in most CTCL patients, especially in those with early stage disease. Side effects of bexarotene are manageable and reversible.

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FREQUENCY AND IMPACT OF CHRONIC GRAFT VERSUS HOST DISEASE (CGVHD) IN OUTCOME OF 822 PATIENTS WHO UNDERWENT ALLOGENEIC BONE MARROW TRANSPLANTATION IN TEN YEARS PERIOD

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Introduction. cGVHD is a common complication occurring after allogeneic hematopoietic stem cell transplant (HSCT) and is the leading cause of non-relapse mortality and morbidity affecting long term survivors. In this presentation we studies incidence and impact of cGVHD in outcome (survival and quality of life) for 822 pts underwent allogeneic HSCT. *Material and method.* From March 1998 to December 2007 (141 months period) 822 pts, with hematologic malignancies (HM): 622 and non-malignant disorders (NMD): 200, underwent allogeneic HSCT (from HLA-identical sibling donors: 816; pheno identical: 6). For study of frequency and impact of cGVHD on survival only 616 pts are appraisable (HM: 495, NHD: 121) (excluded 206 pts: early death < day 100 or early relapse); median age 27 years (4,5 to 59); sex ratio (M/F) 1,23; two conditioning regimens with chemotherapy alone was used: Myeloablative: 426 and non myéloablative: 190; GVHD prophylaxis associated cyclosporin and methotrexate or cyclosporin and mycophenolate mofetil for non myeloablative regimen; Peripheral blood stem cell (PBSC) were used to 585 pts and bone marrow transplant (BMT) to 31 pts ; acute GVHD occurred in 122 pts with 69 grade II-IV and median follow-up 66 months (4-134). cGVHD occurred in 344 pts (55,8%) with median of follow-up 61 months (4-133). To evaluate quality of life (QoL) based on one simple question to pts: do you feel well in live after graft when handicap is absent; all handicap are considered as bad QoL. Only pts who survive above one year are enrolled: 518 pts (HM: 404, NMD: 114) with median age 22 years (4,5 to 59); sex ratio (M/F) 1,44; Myeloablative conditioning regimen : 362 and non myéloablative: 156; GVHD prophylaxis as above; PBSC were used to 500 pts and BMT to 18 pts; cGVHD occurred in 311 pts and no cGVHD in 207 pts; median of follow-up 62 months (13-133). At December 2009 maximal follow up is 141 months and minimal 24 months. *Results.* cGVHD occurred in 344 pts (55,8%): 299 HM (60,4 % of HM) and 45 NMD (37,2% of NMD); with 218 extensive (35,3%) and 126 limited (20,5%). Using a new NIH classification: 293 classic cGVHD (47,6%) and 51 Overlap (8,3%). The Overall Survival (OS) for all pts is 70% at 11 years; OS is 70,4% and 67,6% in pts with and no cGVHD group respectively with limit signification (P=0,04). A good QoL is found in 357 pts (68,9%) with significant difference (P=18-8) between 165 pts cGVHD group (53%) and 192 no cGVHD group (92,7%); A bad QoL is found in 161 pts (31,1 %) (146 pts cGVHD group (46,9%) and 15 no cGVHD group (7,2%; P=10-8). *Conclusion.* The total incidence of cGVHD in our cohort is similar to literature with less Overlap form and incidence of cGVHD is superior in HM than NMD. OS seems similar in the two groups (with or no cGVHD), but in another hand, cGVHD have an impact in QoL as shown that pts without cGVHD had better QoL.

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IN VITRO PLURIPOTENT PROPERTIES AND CHARACTERIZATION OF UMBILICAL CORD BLOOD MESENCHYMAL STEM CELLS

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Background. In recent years, multi-potential mesenchymal stem cells (MSCs) have become an attractive therapeutic tool because of their unique characteristics, such as their ability to be easily isolated and cultured and their high expansive potential *ex vivo*. Umbilical cord blood (UCB) is well known to be a rich source of hematopoietic stem cells with practical and ethical advantages, but the presence of mesenchymal stem cells (MSCs) in UCB has been disputed and it remains to be validated. *Aims.* Because of their availability property the idea that UCB-MSCs can be an alternative source against bone marrow mesenchymal stem cell well accepted by scientists. In our study we use cord blood (n=5) derived mesenchymal stem cells to identify their proliferation, immunphenotypic, immunogenetic and differentiation properties in a comparison with bone marrow mesenchymal stem cells (BM-MSC). *Methods.* Cells were isolated by ficoll-gradient procedure. Immunophenotyping of UCB-MSCs was performed at P3 with antibodies against human antigens CD13, CD44, CD90, CD146 ve CD166, CD3, CD8, CD11b, CD14, CD15, CD19, CD33, CD34, CD45, CD117 by flow cytometry. For immunhistochemistry cells were stained with CD44, CD105, CD146, alfa-smooth muscle actin, beta tubulin, vimentin, fibronectin, nestin, type I and II collagen, c-Fos, myosinIIa, desmin, osteopontine, osteonectine, osteocalsin, CD23, CD31, CD34, CD45, CD71, VWF, PCNA and pluripotent markers such as nanog and OCT4. _mmunhistochemistry datas were confirmed with gene expressions by RT-PCR. Telomerase activity of UCB-MSC and BM-MSCs were performed and for compare proliferation capacity of both cells MTT assay was used. In addition cells at P3 induced osteogenic, adipogenic, myogenic and neurogenic differentiation with appropriate culture conditions. *Results.* UCB-MSCs displayed important embryonic stem (ES) cell characteristics including OCT-4 Rex-1, FoxD, Sox-2, Nanog, SSEA-4. UCB-MSCs also expressed mesenchymal stem cell antigens including CD13, CD44, CD90, CD146 ve CD166, but were negative for CD3, CD8, CD11b, CD14, CD15, CD19, CD33, CD34, CD45, CD117 ve HLA-DR. All of these protein levels were confirmed with gene expressions by RT-PCR. However, telomerase enzyme activity of UCB-MSCs was higher than BM-MSCs. *Conclusions.* Our results demonstrate that, because of UCB-MSCs high telomerase enzyme activity, high potential for expansion and differentiation properties can make them an important resource in regenerative medicine. We considered that , this study will shed light as an important research for future studies of UCB-MSC.

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DIAGNOSTIC VALUE OF CYTOKINE NETWORKS DURING POST-TRANSPLANT COMPLICATIONS

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Backround and Objectives. Complications such as bacterial, fungal and viral infections, veno-occlusive disease, graft-versus-host disease, rejection and relapse are compromise survival following allogeneic and autologous stem cell transplantation (SCT). Although the analysis of a single cytokine is not predictive for any of these problems, concurrent assessment of several serological inflammatory markers may reveal patterns that can be used in clinical situations. Therefore, we measured IL-1β, sIL-2, IL-6, IL-8, IL-10, TNF-α, procalcitonin (PCT) and Ferritin in pediatric patients undergoing stem cell transplantation. *Design and methods.* Maximum values of IL-1β, sIL-2, IL-6, IL-8, IL-10, TNF-α, procalcitonin and ferritin were prospectively analyzed during clinical events post-transplant (range 15 - 245 days) in a cohort of 81 pediatric patients (median age of 7 years) with hematologic malignancies, and non-malignant diseases undergoing allogeneic (in total 74 patients) and autologous (7 patients) hematopoietic SCT. *Results.* PCT, ferritin, IL-6 and IL-1β were strongly correlated with the severity of systemic reaction during infection. An increase of IL-8 was observed in the early phase of sepsis. Upon rejection there was a clear increase of IL-6, TNF-α, PCT and ferritin. An increase of IL-6, PCT and ferritin strongly correlated with the occurrence of veno-occlusive disease, while an increase in ferritin and TNF-α accompanied acute liver GvHD. IL-10 showed a significant increase in acute intestinal GvHD. Interpretation and *Conclusions.* The diagnostic values of IL-1β, sIL-2, IL-6, IL-8, IL-10, TNF-α, PCT, and ferritin may be useful in studies which compare the severity of post-transplant complications. Key factors in this regard are the increase of IL-10 in the acute intestinal GvHD, the increase of TNF-α and ferritin in acute liver GvHD, and the rejection after hematopoietic SCT. Although some patterns could be identified, analysis of more cytokines will be needed to increase the predictive value of these patterns.

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TRANSPLANTATION IN CHILDREN WITH LEUKEMIA WHO LACK HLA-MATCHED RELATED DONORS: CHOICE BETWEEN SEROLOGICALLY MISMATCHED UMBILICAL CORD BLOOD OR ALLELE-MATCHED OR -MISMATCHED UNRELATED DONORS?

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Backgroud: The choice of the best donor for hematopoietic stem cell transplantation (HSCT) is often difficult for pediatric patients lacking HLA-matched related donors (MRDs). *Aims.* The aim of this study was to compare the clinical outcomes of allele-matched (M)-unrelated donor (UD) or -mismatched (MM)-UD transplantation and those of unrelated umbilical cord blood (UCB) transplantation in children with leukemia. *Methods.* We retrospectively evaluated 34 children with leukemias transplanted from UDs at Chonnam National University Hospital between Jan., 1996 and July, 2009. And the results were compared with those from 35 MRD transplants. All UCBs were typed for HLA-A, -B, and -DR at antigen levels. UDs were matched based on allele typing of HLA-A, -B, -C and -DRB1. Allele-MM UDs were accepted for transplantation only when they were 6/6 matched at antigen levels (HLA-A, -B, and DRB1). *Results.* The numbers of transplants were as follows: Allele-M-UD, 10; MM-UD, 13; UCB, 11. In MM-UD group, one allele locus was mismatched in 7, two loci in 5, and three in 1. Most UCBs (n=9) were one antigen mismatched. UCB units were chosen primarily based on cell dose. Median day to absolute neutrophil count (ANC) ≥ 0.5×10^9/L was 15 in MRD, 16 in M-UD, 18 in MM-UD, and 21 in UCB group (P=.01). Median day to platelet ≥20×10^9/L was 19, 23, 30 and 45, respectively (P=.003). Acute graft-versus-host disease (GvHD) of grade II-IV was found in 11.4%, 30.0%, 15.4%, and 36.4%, respectively (P=.124). The incidence of chronic GvHD was 20.3%, 27.1%, 37.7%, and 28.6%, respectively (P=.403). The 5-year Kaplan-Meier overall survival (OS) rate was 77.1% for MRD, 53.8% for M-UD, 71.8% for MM-UD, and 45.5% for UCB (P=.050). MRD group showed higher OS and LFS rate than UCB group (P=.013, P=.017, respectively). However, the OS rate of M- and MM-UD together (65.7%) was not different from that of MRD group (77.1%, P=.270), or from that of UCB (45.5%, P=.153). The cumulative incidence of relapse at 5 years was 18.6%, 25.0%, 10.0%, and 0%, respectively (P=.530). The cumulative incidence of 100-day transplant-related mortality (TRM) was 2.9%, 22.2%, 7.7%, and 36.4%, respectively (P=.011). *Conclusion.* The outcome of HSCT in children using alternative donors has improved significantly in recent years, now approaching to that from MRDs. MM-UD transplants showed at least comparable survival to M-UD transplants, if serologically 6/6 matched. The benefit of low relapse rate in UCB group was offset by a high TRM in early post-transplant period, probably secondary to a slower engraftment. UD transplant, even mismatched, should be pursued in patients who lack MRD. Improvement in the selection of UCB units and supportive measures should result in a better outcome in UCB transplantation. These results justified the simultaneous search of unrelated BM donors and unrelated UCB units when a child with acute leukemia is in need of an alternative transplants. The decision should be based on the number of HLA disparities, the urgency of the transplant, and cell contents of the graft.

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A BENEFICIAL ANTI-VIRAL IMMUNE RESPONSE RESTRICTED BY ALLO-HLA

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Background. Recognition of an antigen in the context of allo-HLA molecules is not always harmful. Tumor associated self antigens (TAAs) recognized in the context of allo-HLA can be targeted for therapeutic purposes. Several research groups have investigated whether they could generate high avidity T cells against TAAs presented in allo-HLA molecules by stimulating T cells with HLA mismatched stimulator cells loaded with TAAs, thereby circumventing self tolerance. We recently showed that high avidity PRAME specific T cells which are allo-HLA restricted, could develop *in vivo* exerting high reactivity against multiple tumors with very limited recognition of normal tissues *in vitro*. We wondered whether beneficial T cell responses against viral antigens recognized in the context of allo-HLA could also develop *in vivo* after HLA mismatched SCT. *Aim of the study.* We investigated whether anti-viral immune responses restricted by allo-HLA can develop *in vivo* after HLA mismatched SCT. *Methods.* PBMCs of patients collected at different time points after HLA mismatched SCT were stimulated with EBV-LCLs loaded with CMV pp65 or IE-1 overlapping peptides for 4 hours after which intracellular IFN-γ was measured in CD4 and CD8 T cells. The EBV-LCLs were derived from patient, donor, or HLA typed individuals expressing one of the shared or non-shared HLA alleles of patient and donor. In addition, tetramer stainings were performed. *Results.* In a patient (HLA A*0220 A*2402 B*0801 B*1401) transplanted with a haploidentical donor (HLA A*0220 A*0101 B*0801 B*1401) we observed shortly after SCT at the time of CMV reactivation, CMV specific immune responses restricted by the shared HLA-B8 molecule as well as CMV responses restricted by donor non-shared HLA-A1. The immune response restricted to the shared HLA-B8 increased in time, and was present still 3 years after SCT. In contrast, the HLA-A1 restricted immune response decreased in time, and was undetectable 1 year after SCT. Interestingly, at the time of reactivation a CMV-IE1 immune response restricted by patient non-shared HLA-A24 was observed. The virus specific T cells were of donor origin. The HLA-A24 restricted response increased over time and persisted for several years after SCT. In addition, both the HLA-A24 as well as HLA-B8 restricted response correlated with viral load. T cells were clonally expanded and demonstrated IE1 peptide specific HLA-A24 restricted IFN-γ production, whereas A24 restricted EBV-LCLs without CMV-IE1 peptide were not recognized, indicating that this response was solely IE1 peptide specific. *Conclusion.* In a haplo-identical transplanted patient an immune response was found against a CMV-peptide presented in allo-HLA which substantially contributed to the immune response leading to the clearance of CMV viral reactivations next to an immune response via a shared HLA-molecule. These results demonstrate that beneficial anti-viral immune responses restricted by allo-HLA molecules can develop *in vivo* after HLA mismatched SCT.

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THE IL-12B 3'UTR (-1188 A/C) POLYMORPHISM IS ASSOCIATED WITH SURVIVAL OUTCOME OF HEMATOPOIETIC STEM CELL TRANSPLANTS

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Introductio: Interleukin-12 (IL-12) is a pro-inflammatory cytokine which mediates a myriad number of roles , among which includes the differentiation of naïve $CD4^+$ T-cells to Th1 lymphocytes and the stimulation of Interferon-γ (IFN) production from T and natural killer (NK) cells. IL-12 is a 75 kDa heterodimer, composed of p35 and p40 subunits. Several polymorphisms have been identified at the IL-12 p40 gene (IL12B), of which the IL12B 3'UTR (-1188A/C) polymorphism has been reported to influence IL-12 and IL-10 production. We sought to identify if this polymorphism had an impact on the outcome of allogeneic hematopoietic stem cell transplantation (HSCT), in view of its potential role in mediating cell mediated and innate immunity. *Materials and Methods.* Thirty-one patients undergoing HSCT for various hematological conditions were recruited. Pre-transplant peripheral blood samples were obtained and subjected to genotyping for the cytokine IL12B 3'UTR (-1188A/C) polymorphism, among others, by PCR-SSP using the Cytokine Genotyping Kit (Invitrogen, WI, USA). HSCT survival outcomes were compared for the different genotypes by Kaplan-Meier analysis and multivariate Cox regression. *Results.* Gene frequencies of the biallelic IL12B 3'UTR (-1188A/C) polymorphism in the study population was not significantly different from the expected Hardy Weinberg proportions with 8/31 (26%) subjects AA homozygous, 15/31 (48%) AC heterozygous and 8/31(26%) CC homozygous. IL12B 3'UTR polymorphism was significantly associated (P=.030) with overall survival (OS) following HSCT. Subjects who were AA homozygous had a median survival of 282 days (95% CI: 0 - 610 days) in comparison to 1,155 days (95% CI: 591 - 1,711 days) for AC and CC subjects combined. Analysis for other factors which may have contributed to poor post-HSCT OS revealed that disease status with patients transplanted while in blast crisis of chronic myeloid leukemia or after first or second relapse of acute myeloid leukemia had significantly (P=.032) reduced OS. IL12B 3'UTR polymorphism however remained significant for OS after adjustment for disease status using a multivariate Cox proportional hazards model. *Conclusions.* Our study demonstrates that the IL12B 3'UTR (-1188A/C) polymorphism may play a role in the survival outcome of patients undergoing HSCT. Individuals AA homozygous at this position have been shown to have reduced IL-12 secretion from stimulated peripheral blood mononuclear cells. This may have relevance in our cohort of patients who were AA homozygous, as the cause of death in all of them were attributed to either infection or disease relapse but not graft *vs.* host disease. Attenuation of IL-12 response in AA homozygote patients may contribute to suboptimal graft *vs.*

leukemic response leading to disease relapse, as well as lowered cell mediated immunity leading to risk of viral infections.

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WHERE HAVE ALL THE ABSTRACTS GONE? PUBLICATION BIAS IN BLOOD AND MARROW TRANSPLANTATION

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Background. Only a small proportion of abstracts lead to full publication, leading to publication bias. Clinical and "positive"studies are more likely to be published than other abstracts. This issue has not been examined in the hematology or blood and marrow transplantation (BMT) literature. *Aims.* We intend to review the rate of publication of abstracts presented at the Canadian Blood and Marrow Transplant Group (CBMTG) Annual Meetings, and determine factors associated with publication. We hypothesized that as BMT centres in Canada are based at large, academic centres, the proportion of abstracts leading to publication will be high. *Methods.* All abstracts presented at the CBMTG Annual Meeting in 2002, 2004, and 2006 were reviewed and categorized by: study type; funding source; single or multi-centre; form of presentation; "positive"or "negative", using the authors' definition. To determine publication, each reference was searched on multiple databases (MEDLINE, EMBASE, Web of Science, and CINAHL) by first, second and final authors. Two authors undertook abstract categorization and searching, and disagreements were resolved by consensus. *Results.* 141 abstracts were reviewed, of which 43 were published (30.4%); this proportion is comparable to other medical specialties. 21 studies were published from 2002 (36.8%), as compared to 12 from 2004 (24.0%) and 10 from 2006 (29.4%) (P=0.35). Clinical studies (retrospective or prospective) were more likely to be published than non-clinical studies (P=0.014). Number of centres involved and positive results were not associated with publication likelihood. Funded studies were more likely to be published (P=0.009). Finally, oral presentations were more likely than posters to be published (P=0.004). *Conclusion.* Publication bias exists at the CBMTG meeting at a rate similar to that of other medical disciplines. Studies with clinical outcomes, ones that were externally funded, and those presented orally were more likely to be published. Efforts to encourage full publication of scientific abstracts should be sought.

Table 1. Study results.

Predictors of Abstract Publication	Rate of Publication	Significance
Form of Presentation		
Oral	59.1%	p = 0.004
Poster	25.2%	
Funded Study		
Funded Study	70.0%	p = 0.009
Non-Funded Study	27.5%	
Study Type		
Clinical	38.4%	p = 0.014
Non-Clinical	18.2%	
Number of Centres Involved		
Single Centre	27.7%	p = 0.130
Multiple Centres	45.5%	
Study Results		
Positive	37.7%	p = 0.186
Negative (or not-stated)	26.1%	
Year		
2002	36.8%	p = 0.350
2004	24.0%	
2006	29.4%	

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VALIDATION OF THE DNA DYE 7AAD FOR THE DETECTION OF LOSS OF VIABILITY IN STORED HAEMATOPOIETIC PROGENITOR CELLS: A COMPARISON WITH OTHER VIABILITY PROBES AND CORROBORATED BY LONG TERM CULTURE

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Haematopoietic Progenitor Cells (HPC) are stored at 4°C up to 72 hours or cryopreserved and their subsequent viability is central to the success of HPC transplantation. Because levels of apoptosis and necrosis in thawed HPC harvests have been reported to be high (>50%) and testing of cryopreserved products is not possible before infusion, small pilot samples were cryopreserved or stored at 4°C. The initial aim of this study was to validate the use of pilot samples by comparison with samples taken from the harvest at infusion using flow cytometry of the fluorescent probe 7 amino-actinomycin D (7AAD) which reportedly discriminates three populations: negative (viable), dim (apoptotic) and bright (necrotic), in the CD34+ve HPCs. The second aim was to show that 7AAD was as sensitive as other viability probes; - Annexin V, - 3, 3'- dihexylocarbocyanine iodide (DiOC6(3)), - AldefluorTM, - Syto16, and that there was a direct relationship between staining with 7AAD and the ability of HPC to produce cobblestone areas and granulocytes into the supernatant when co-cultured on normal bone marrow derived stromal cells for a minimum of 5 weeks (functional assay). To perform the functional assay, normal bone marrow cells were cultured for between 6 and 14 weeks after which they were passaged and re-seeded into αMEM. These cultures were taken through 4 further passages before the medium was replaced with a long term culture style medium. The stromal layers were then innocculated with either fresh or cryopreserved HPC. There was no increase in 7AAD uptake in pilot samples, P=0.22 making them an effective surrogate. Storage of HPC for 72 hours at 4°C, which is the generally accepted expiry, only showed a significant change in one of the probes measured, AldefluorTM, and it has been previously reported (by other authors) that a reduction in the proportion of AldefluorTM bright HPC can be correlated with prolonged platelet engraftment times. The functional assay showed that there was no significant difference between the production of cells into the supernatant after 5 weeks culture in unstored (fresh) and cryopreserved HPC P=0.34. The cell production from cultures of HPC stored between 24 and 72 hours at 4°C was increased, possibly as a result of loss of monocytes which may be inhibitory in this model system. Co-cultured cryopreserved samples that showed high 7AAD staining either did not survive for 5 weeks or showed minimal cobblestone and cell production. There was a decrease in the proportion of Aldefluor bright HPC in cryopreserved pilot samples by comparison with unstored HPC, P=0.08. In conclusion, AldefluorTM may be a more sensitive probe than 7AAD for loss of viability in products stored at 4°C and cryopreserved however this was not corroborated by long term culture studies. The loss of AldefluorTM bright cells may be measuring the loss of progenitors that do not contribute to cell production in this long term culture model. Assay of 7AAD uptake by HPC is a simple and cost effective method of measuring HPC viability that correlates well with the results of long term co-culture.

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LONG -TERM OUTCOME AFTER BONE MARROW VERSUS PERIPHERAL BLOOD STEM CELL TRANSPLANTATION: A RETROSPECTIVE SINGLE- CENTRE ANALYSIS

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Introduction. Peripheral blood (PB) is used more frequently as a source of stem cells (SC) for allogeneic transplantation. Despite many years of experience, the effect of SC source on the clinical outcome in allogeneic transplantation, as treatment modality of hematological malignancies is not yet well established. *Aim.* We have compared results of allogeneic peripheral blood SC transplantation (PBSCT) with bone marrow transplantation (BMT) in the treatment of hematological malignancies with respect to engraftment, transfusional needs, frequency and severity of immediate (mucositis, acute Graft *vs.* Host Disease - aGvHD) and delayed (chronic GvHD - cGvHD) complications of the procedure, transplant related mortality (TRM), relapses and overall survival (OS). *Methods.* We have analyzed 158 patients (pts.), median age was 29 (range 9-57), 64 females, 94 males with different hematological diseases (AML - 39, ALL - 47, CML - 32, MDS - 10, HL - 2, MM- 3, Granulocytic sarcoma -3, SAA-22) in whom we performed allogeneic stem cell transplantation (SCT) in our center from 1989. until 2009. Pts. were divided into two groups according to the SC source: the first group with BMT-74 pts. and second group with PBSCT- 84 pts. Each patient had HLA identical sibling donor. SC from bone marrow (BM) were collected by standard procedure and from the PB by one procedure of "large volume apheresis" after the application of rHuG-CSF 5-12 μg/kgbm during 5 days. All pts. have received unmanipulated suspension of SC. Conditioning regimens were applied according to primary disease, GvHD prophylaxis were consisted of combination of Cyclosporine A and Methotrexate. *Results.* Engraftment, according to number of polymorphonuclear (PMN) and platelets (Plt) were significantly faster for 6 days (P<0.001) in the group of pts. treated with PBSCT comparing to BMT group. Needs for the transfusional support (red blood cells- RBC and Plt) were significantly higher in the BMT group (P<0.01). Those pts. had more frequently oropharingeal mucosi-

tis grade 3/4 (33.3% *vs.* 10.0%, P<0.05). There were no significant differences in the frequency of aGvHD and cGvHD between these two groups. Pts. who were undervented PBSCT had more frequently extensive cGvHD in comparison to those pts. treated with BMT (29.1% *vs.* 11.29%, P<0.05). Source of SC had no significant influence on TRM (21,62% *vs.* 23,8%, P=0,64, ns), incidence of relapses (21.6% *vs.* 29.7%, P=0.32, ns). Pts. in whom SC source was BM had significantly better OS then other group (log-rank 2.33, P<0.05). *Conclusion.* PB as a source of allogeneic hematopoietic SC gives better mononuclear cells (MNC) yield and faster engraftment with consequently decrease of immediate transplantation related complications, with positive influence on economic aspect of treatment. However, allogeneic PBSCT was associated with more frequent extensive cGvHD, while we haven't noticed influence of SC source to TRM and relapses. In our series, better OS had patients with BMT but future investigations are needed.

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HUMAN T-CELL LYMPHOTROPIC VIRUS TYPE 1 (HTLV-1) PROVIRAL DNA LOADS IN THE LONG SURVIVORS WITH ADULT T-CELL LEUKEMIA/LYMPHOMA (ATLL) AFTER ALLOGENIC HEMATOPOIETIC CELL TRANSPLANTATION

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Background. Adult T-cell leukemia/lymphoma (ATLL) is a highly aggressive hematological malignancy caused by a human T-cell lymphotropic virus type 1 (HTLV-1), and has a poor prognosis. Recently, some patients achieve long survival after allogenic hematopoietic cell transplantation (allo-HCT). However, HTLV-1 proviral DNA loads in the long survivors with ATLL after allo-HCT are not clarified. *Aims.* By analyzing HTLV-1 proviral DNA loads in the long survivors with ATLL after allo- HCT, we clarify the association between risk of ATLL relapse and HTLV-1 proviral DNA loads. *Methods.* We analyzed HTLV-1 proviral DNA loads in peripheral blood in 10 ATLL patients survived over 24 months after allo-HCT in our hospital. Of these, 9 had acute type, 1 had chronic type. Disease status at HCT were 8 remission, 2 non-remission. Eight cases had conventional stem cell transplantation (CST) and 2 cases had reduced-intensity stem cell transplantation (RIST). Six patients received stem cells from bone marrow, 4 patients received from peripheral blood. Six patients underwent HCT from HLA-identical siblings, 1 donor having anti-HTLV-1 antibodies, and 4 from unrelated HLA-identical donors. We compared proviral loads in the 9 patients with remission for long time (non-therapy over 6 years) after chemotherapy and in the172 asymptomatic HTLV-1 carriers. The HTLV-1 proviral loads in peripheral blood mononuclear cells (PBMCs) were measured by quantitative real-time PCR (RT-PCR) with primers specific for HTLV-1 pX. *Results.* The mean value of HTLV-1 proviral DNA loads were 0.09 copies/100 PBMCs in the allo-HCT patients and 6.28, 3.72 copies/100 PBMCs in the patients with remission after chemotherapy in the asymptomatic HTLV-1 carriers respectively. Proviral loads in the allo-HCT patients were significantly lower than in the patients with remission after chemotherapy (P=0.03), lower than in the asymptomatic HTLV-1 carriers (P=0.05). Proviral loads in the long suvivors with ATLL after allo- HCT rapidly decreased after HCT and extremely low loads maintained. *Summary/Conclusions.* Our results suggest that risk of ATLL relapse is lower in the long survivors after allo-HCT than in the patients with remission after chemotherapy and in the asymptomatic HTLV-1 carriers. Further studies are needed to clarify the association-between relapse and HTLV-1 proviral DNA loads.

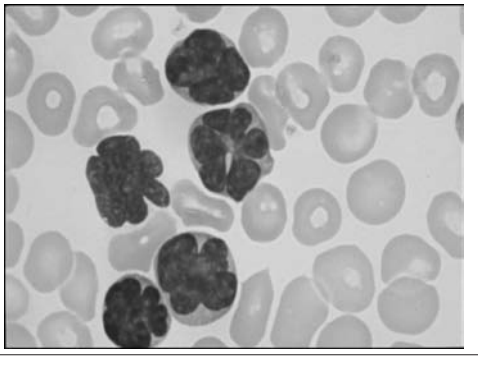

Figure.

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WITHDRAWN BY AUTHOR

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A THEORETICAL MODEL FOR CELL DYNAMICS AFTER ALLOGENEIC STEM CELL TRANSPLANTATION IN ACUTE LEUKEMIA

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Background. The beneficial effect of allogeneic stem cell transplantation (allo-SCT) is two-pronged: on one hand the conditioning regimen is intended to destroy the recipient's hematopoiesis, which at the time of the transplant contains both normal and leukemic stem cells and on the other hand the donor-derived T-lymphocytes exert a long-lasting immunologic graft-versus-leukemia effect (GVL), aimed at destroying those recipient cells that have withstood the effect of the conditioning regimen. It has also been demonstrated that by reducing the conditioning regimen dose and enhancing the GVL effect by transplanting high quantities of donor derived stem cells and T-cytotoxic lymphocytes, the outcome of the transplants may be improved. *Aims.* Understanding the cell dynamics during and after transplantation is essential for devising new strategies, aimed at improving both the efficacy and safety of allo-SCT. *Methods.* We used mathematical modeling with the help of dynamic systems and we performed numerical simulations with Maple 11.

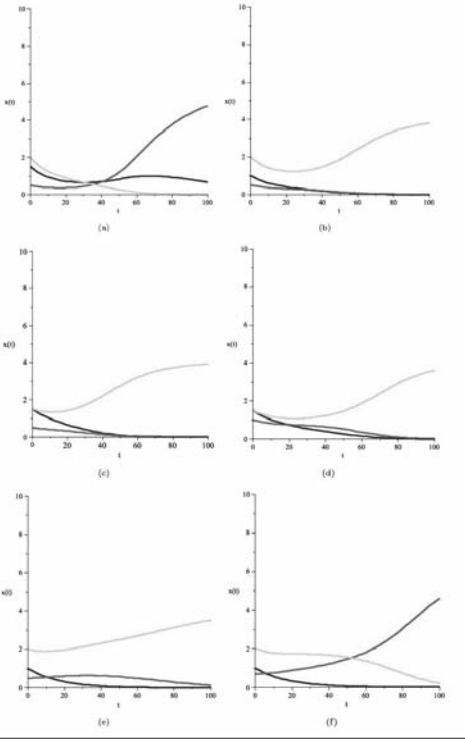

Figure.

Results. We obtained a basic mathematical model expressed by a system of ordinary differential equations tracking the time evolution of three cell lines after allo-SCT: normal host cells, leukemic host cells and donor cells. The evolution of these cell populations is one of competitive type and depends upon kinetic parameters: intrinsic growth, death and microenvironment sensitivity rates of each of the populations and parameters measuring the intensity of the cell-cell interactions between these populations. By numerical simulations we show that the evolution can ultimately lead either to the normal hematopoietic state with the donor cells eliminating the host cells, or to the leukemic state characterized by the proliferation of the leukemic line and the suppression of the other cell lines. One state or the other is reached depending on cell-cell interactions (anti-host, anti-leukemia and anti-graft effects) and initial cell concentrations at transplantation. The figure presents in (a)+(b) numerical simulations for identical anti-host, anti-leukemia and anti-graft effects and two sets of initial (at time t = 0) cell concentrations. In case

(a), in time, normal cell population (blue line) and donor cell population (green line) approach 0 while the cancer cell population (red line) becomes dominant; in case (b) host normal and cancer cell populations tend to 0 while donor cells restore the normal hematopoietic state. In (c)+(d) we assume that anti-host and anti-leukemia effects are intense while anti-graft effect is weak. Then the transplant is successful with high concentrations of host cells and relatively low concentration of donor cells. Next in (e)+(f) we assume strong anti-host effect and weak anti-leukemia and anti-graft effects. This makes the transplant result very sensitive to the initial leukemic cell concentration. *Conclusions.* Although simple, our model takes into account essential biological properties and processes such as cell growth and death, cell-environment and cell-cell interactions. It allows us to perform numerical simulations to investigate the impact of the intensiveness of cell-cell interactions and of initial cell concentrations on transplant success. The model can also be used to control the post-transplant patient evolution and provide better guidance towards the recovery of normal hematological status.

1643

INFLUENCE OF NEONATAL SEX, WEIGHT AND STROMAL CELL-DERIVED FACTOR 1 POLYMORPHISM ON CD34+ CELL CONCENTRATION IN UMBILICAL CORD BLOOD

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Background. Hematopoietic stem cells (HSC) transplantation over the last decade has been marked by frequent use of umbilical cord blood (UCB) as an alternative source of donor's HSC. Therefore, a main limitation to increase the use of UCB in adults' transplantation is related to UCB's low total nucleated cell (TNC) and $CD34^+$ cell dose/kg recipient body weight. *Aims.* For this reason many attempts are evaluated for selecting units best suited for transplantation in the aim of improving its efficiency in adults and reduce the cost of processing. In this study the impact of sex, weight of neonates and stromal derived factor 1α (SDF1 α) polymorphism on the yield of UCB units has been evaluated. *Materials and methods.* Cord blood was collected in Macopharma bags containing 21 ml of citrate phosphate dextrose anticoagulant, by trained midwives before the delivery of the placenta. CD34+ cell concentration in placental blood was routinely determined by flow cytometry only in units exceeding 100 g for possible banking. The number of TNC was determined by Cell-Dyn 3000. Seven hundred and fifty cord blood samples were analysed with respect to the sex of neonates and the collected volume. One hundred and eighty neonates were investigated for SDF1 polymorphism (homozygoties A/A, G/G, or heterozygoties A/G), determined by a particular tetra PCR after obtaining parental consent. *Results.* Ninety percent of UCB samples range between 14-75 $CD34^+$ cells/mm^3 (median=32) and in contrast with the TNC number, $CD34^+$ cells concentration did not respect the Gauss distribution. Our results in 750 UCB units (male N= 365 and female N= 385) showed that male neonates UCB were richer in CD34+ (P<0,001) though the female ones were richer in total nucleated cells (TNC) (P=0,01); a slight correlation between $CD34^+$ cells concentration and UCB sample weight (P<0,01) could be attributed to a higher weight in male neonates. Furthermore, using a particular tetra PCR performed in 180 neonates and considering SDF1 α polymorphism, no difference between A/A, G/G, A/G allelic combination was found. *Conclusion.* These data emphasize the lack of predictive factors influencing the $CD34^+$ and TNC concentration in UCB units before processing. Indeed, our results suggest that sex and weight of neonates influence $CD34^+$ cell content as it has been hypothesized by previous studies. Moreover, genetically determined polymorphism in SDF1α axis did not seem to be relevant and useful for the a priori selection of UCB units exhibiting a better hematopoietic potential.

1644

ROLE OF ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANTATION FOR PATIENTS WITH RELAPSED/REFRACTORY ACUTE MYELOID LEUKEMIA

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Background. Patients with AML can rarely get long-term survival with chemotherapy alone when they are in primary-refractory status or fail to achieve remission with reinduction (RI) after relapse. *Aims.* It remains unknown whether allogeneic hematopoietic cell transplantation (alloHCT) can induce a durable remission for patients with detectable disease at the time of alloHCT. *Methods.* Patients who received alloHCT in their relapsed status after the 1st remission (CR1) or in refractory status between 1996 and 2009 at the Asan Medical Center were analyzed. Those who relapsed were categorized into 3 groups; relapsed and obtained remission with RI (REL-S), relapsed and did not receive RI (REL-U), relapsed and did not obtained remission with RI(REL-R). Those who were primarily refractory were categorized as a separate group (REF). *Results.* Median age of fifty patients (male: female = 30: 20) was 35.5 (range, 17-63) years. Number of patients in each group was 25(REL-S), 12(REL-U), 4(REL-R) and 9(REF). Among 41 patients except those in 'REF', median time between CR1 and relapse ranged from 5.5 for 'REL-U' to 15.3 months for 'REL-S'. Type of conditioning and immunosuppressant were not different significantly among 4 groups. Engraftment was achieved in 96% of patients and BM examination was performed on a median day 35 (range, 15-197) from the cell infusion. Outcomes in each group were listed at Table 1. When patients in 'REL-S' were analyzed in combination with those in 'REL-R', the overall survival (OS) of 29 patients was similar to that in 'REL-U' (21.8 *vs.* 20.7 months, P=0.562). Among those in 'REL-U', age (<40 *vs.* ≥40 years) and BM blast percentage (<12.5 *vs.* ≥12.5%) were associated with OS in univariate analysis. *Conclusion.* Although most of patients in 'REL-U' failed to achieve a durable remission, OS could be improved and selected patients could obtain a long-term survival. The role of alloHCT needs to be investigated further for those in 'REL-R'.

Table 1.

	Total (n=50)	REL-S (n=25)	REL-U (n=12)	REL-R(n=4)	REF(n=9)
Complete remission	40(80%)	23(92%)	10(83%)	0	7(78%)
Duration of response (months)	-	15.4(2.5-164.0)	2.6(0.7-101.9)	-	6.3(1.1-21.6)
Event rate among CR	25(63%)	13(57%)	9(90%)	-	3(43%)
Death without relapse	3(8%)	3(13%)	0		0
Relapse	20(50%)	9(39%)	8(80%)		3(43%)
Performance of DLI	2(5%)	1(4%)	1(10%)		0
Status of disease at last f/u					
No evidence of disease	28(56%)	19(76%)	3(25%)	1(25%)	5(56%)
Persistent disease	20(40%)	5(20%)	9(75%)	3(75%)	3(33%)
Event-free survival (months)	8.9(1.5-77.7)	17.4(4.6-77.7)	5.2(1.5-9.1)	6.0(3.7-8.2)	4.7(1.8-26.4)
Overall survival (months)	17.4(1.8-165)	48.0(4.6-165)	20.7(2.9-103)	6.6(6.0-11.1)	17.4(1.8-26.4)

1645

TACROLIMUS/SIROLIMUS AS A GVHD PROPHYLAXIS AFTER HIGH RISK ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION: COMPARISON WITH CONVENTIONAL REGIMENS

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Background. Graft-versus-host disease (GVHD) remains a major cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (HSCT). Transplantation from donors other than HLA-matched siblings is limited by a substantial risk of severe GVHD using conventional cyclosporine (CsA) or tacrolimus based prophylaxis. The combination of sirolimus, an inhibitor of the mammalian target of rapamycin (mTOR) and tacrolimus has resulted in a low incidence of acute GVHD and reduced transplant-related toxicity in several studies. However, in a recent study, administration of sirolimus was discontinued earlier than planned due to its toxicity. Other studies showed sirolimus was associated with an increase risk of some serious complication, such as thrombotic microangiopathy and veno-occlusive disease. *Aims.* We sought to confirm the efficacy and safety of sirolimus in combination with tacrolimus for GVHD prophylaxis compared to conventional regimens (CsA/MTX or tacrolimus/MTX) in mismatched related or unrelated donor transplantation. *Methods.* 48 patients received filgrastim-mobilized peripheral blood stem cells after conditioning regimen consisting of busulfan and fludarabine±antithymoglobulin. And patients were treated with 3 different regimens for GVHD prophylaxis: CsA/MTX (n=17), Tacrolimus/MTX (Tac/MTX; n=18), and Tacrolimus/Sirolimus (Tac/Sir; n=13). *Results.* All patients engrafted, with a median time to neutrophil engraftment of 12 days. The incidence of grade II-IV acute GVHD in Tac/Sir group was lower than other groups (15.4% for Tac/Sir group *vs.* 42% for CsA/MTX group, and 50% for Tac/MTX group; P=0.44). Significant factor associated with increase the risk of grade II-IV acute GVHD were conditioning regimen includ-

ing anti-thymocyte globulin and unrelated donor compared to mismatched sibling donor. The day-100 non-relapse mortality (NRM) was similar in the each group (7.7% for Tac/Sir group, 5.5% for Tac/MTX group, and 11.8% for CsA/MTX group). Veno-occlusive disease of liver was developed in one case with CsA/MTX, and two cases with Tac/MTX. However, there was no patient who experienced thrombotic microangiopathy. Although the incidence of CMV or EBV reactivation in Tac/Sir group was higher than other group (61.5% *vs.* 41.8% for CsA/MTX group, and 38.9% for Tac/MTX group), there was no case with opportunistic viral disease which led to death. Most of cases with viral reactivation developed within the day-100 after HSCT. To evaluate the influence of GVHD prophylaxis regimens on immune reconstitution, $CD4^+$ cells seemed to be lower on days 100 after HSCT in Tac/Sir group without statistical significance. *Conclusions.* The combination of tacrolimus and sirolimus is well tolerated and may be helpful in deceasing the risk of acute GVHD and NRM in case of high risk allogeneic HSCT. However, this combination must be used with caution because of its toxicity and we need a differentiated strategy according to the risk of acute GVHD.

1646

DASATINIB AS SALVAGE THERAPY FOR IMATINIB-REFRACTORY SCLEROTIC CHRONIC GRAFT-VERSUS-HOST DISEASE

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Background. Sclerotic chronic graft-versus-host disease (ScGvHD) is an immune mediated disease resembling systemic sclerosis with limited and disappointing treatment options. Upregulation of platelet derived growth factor-receptor (PDGF-R) intracellular signalling pathway has been proposed as a key mechanism which leads to the pathologic lesions observed in these fibrotic diseases. Imatinib mesylate (IM) inhibits PDGF-R kinase activity, and has been proposed as a novel therapeutic alternative for ScGVHD. Several case reports and two small series have shown that up to 50-79% of ScGVHD patients can improve following IM treatment. Conversely, one third to half of these patients failed to respond or was intolerant to IM. Dasatinib is a second-generation PDGF-R kinase inhibitor with a greater inhibitory potency and proven clinical efficacy in the treatment of CML patients refractory or intolerant to IM. *Aims.* We hypothesized that dasatinib may be an effective therapeutic alternative for patients with ScGVHD who are intolerant or refractory to IM. *Patients and methods.* We describe here a small series of three patients with extensive ScGvHD who failed IM treatment and went on to receive salvage therapy with dasatinib. Patient #1: female, 58 years, AML, related reduced-intensity conditioning alloSCT. Patient #2: male, 28 years, AML, related myeloablative alloSCT. Patient #3: female, 27 years, AML, matched unrelated myeloablative alloSCT. Histological features of ScGVHD were documented in all three cases (e.g. pandermal collagenosis, panniculitis, loss of rete ridges, dermal apendages and hair follicles). Prior to dasatinib, all patients had failed treatment with systemic corticosteroids, cyclosporine A and mofetil mycophenolate, in addition to IM (#1 and #2 IM-intolerant after eight and three months, respectively, #3 IM-refractory with progressive ScGVHD despite five months on IM). *Results.* Dasatinib was prescribed on compassionate use basis at a starting dose of 50mg daily, was well tolerated, and the dose escalated up to 100mg daily in all cases within 8 weeks from the start. Patients #1 and #2 have achieved partial responses and objectivable clinical recovery (improvement in Karnofsky Performance Status, skin score and thickness, range of motion, reduction in the body surface area involved by non-moveable sclerosis and resolution of skin ulcers) at three months, which continue at seven and five months most recent follow-up, respectively. Patient #1, who also suffered severe lung involvement, showed measurable improvement in pulmonary function tests and at 2-minute walk distance test. Furthermore, dasatinib treatment induced stable disease at three months in patient #3, who had progressed while on treatment with IM (non-moveable sclerosis involving >30% of body surface area, joint contracture predominantly in left lower extremity and Karnofsky Performance Status score 2). During dasatinib treatment, none of the patients presented worsening of any sclerotic area. In addition, the dose of corticosteroids could be reduced >75% to <0.1 mg/kg of oral prednisone in all cases. *Conclusions.* Our small series suggests that Dasatinib, a potent second-generation PDGF-R kinase inhibitor, may be an effective and well tolerated therapeutic alternative for patients with ScGVHD who are intolerant or refractory to IM. The evaluation of its role in this clinical setting needs further investigation.

1647

NOD2/CARD15 VARIANTS ARE NOT A RISK FACTOR FOR CLINICAL OUTCOME AFTER NON-MYELOABLATIVE ALLOGENEIC STEM CELL TRANSPLANTATION

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Background. Single nucleotide polymorphisms (SNPs) in the innate immunity receptor NOD2/CARD15 have been demonstrated to modulate the outcome of allogeneic hematopoietic stem cell transplantation (SCT). The effect of NOD2/CARD15 polymorphism seems to be associated with type of donor (sibling or matched unrelated donor) as well as type of conditioning regimen. *Methods.* We reviewed NOD2/CARD15 SNPs in all donor/recipient pairs of 192 consecutive patients who received non-myeloablative allogeneic SCT at our institution between 2002 and 2006. All patients were treated with fludarabine 30 mg/m^2/day for 3 days followed by 200 cGy total body irradiation (TBI) (n=154) or TBI alone (n=38) and received grafts from HLA-matched related (n=132) or unrelated (n=61) donors. *Results.* NOD2/CARD15 polymorphisms were observed in 36 of 192 (19%) patients and in 35 of 192 (18%) donors. The incidences of acute and chronic graft-versus-host disease (GVHD) were 39% and 49% respectively in patients with NOD2/CARD15 variants *vs.* 51% and 61% in patients with wild type. The relapse rate at three years was 38% in patients with variants and 36% in patients with wild type. The incidence of transplant-related mortality was 22% for patients with SNPs and 21% for patients with wild type. Overall survival at three years was 56% in patients with variants and 64% in patients with wild type NOD2/CARD15. There was no significant impact on clinical outcome (P>0.05, Kaplan Meier and Fine & Gray's test). Conclusion. These data indicate that mutations in the NOD2/CARD15 gene are not a risk factor for clinical outcome in non-myeloablative allogeneic SCT. Therefore, screening for NOD2/CARD15 polymorphisms in patients or donors does not have additional value in patients undergoing non-myeloablative SCT.

1648

SUCCESSFUL MOBILIZATION OF PERIPHERAL BLOOD PROGENITOR CELLS WITH PEGFILGRASTIM

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Background. Pegfilgrastim is a pegylated growth factor with an extented serum hal-life. Many studies have shown its efficacy in chemotherapy induced neutropenia. Although few, some studies suggest that a single dose of Pegfilgrastim can mobilize patients with lymphoma. *Aims.* To evaluate the efficacy and safety of Pegfilgrastim 6mg for mobilizing peripheral blood progenitor cells. To evaluate the engrafment in pts undergoing autologous stem cell transplant (ASCT). *Methods.* Between june 2006 and june 2009, 52 consecutive pts (M = 31, F = 21)with Non Hodgkin's Lymphoma (NHL) (n=42) or Hodgkin's disease (HD) (n=10) in first line or salvage treatment and planned to receive ACST received a single Pegfilgrastim 6 mg injection 24 to 48 hours after the end of chemotherapy. CD34 circulating cells were monitored from day 6 of Pegfilgrastim injection and apheresis were started as soon as the targeted CD34 cells of 10/mm^3 was reached. All atients collected and with chemosensitive disease were then planned to receive high dose chemotherapy (HDT) with BEAM or Z-BEAM (1 pt) and stem cell support followed by standard growth factors from day 5 until ANC count > 1.000/mm^3. The primary end-point of the study was to evaluate the ability to achieve a number of harvested CD34 cells/Kg ≥2×10^6. *Results.* Fifty pts (96%) have been succesfully collected. The median number of CD34 cells collected was 4×10^6/Kg (0.6-30.1) and the median number of apheresis was 2 (1-4). The median number of circulating CD34 cells

at time of first apheresis was 34/mm^3 (10-416). It is worthy of note that 86% of pts (45/52) required only 1 or 2 apheresis. Thirty nine pts (78%) underwent HDT followed by ASCT.All pts recovered from aplasia within a median of 10 days (6-24) without unexpected complications. *Conclusion.* A single injection of Pegfilgrastim 6 mg is safe and effective with 96% of pts succesfully mobilized. Only one or two apheresis are sufficient for the majority of pts (86%).

Updated results will be presented at the meeting.

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EPCR GENE'S HAPLOTYPES AND ACUTE GRAFT VERSUS HOST DISEASES

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Graft-versus-host disease (GVHD) is the major cause of morbility and mortality after allogeneic hematopoietic stem cell transplantation (HSCT). GVHD has traditionally been classified in acute and chronic according to the time of the onset. Acute GVHD (aGVHD) involves skin, liver, gastrointestinal tract, and possibly other organs and its pathophysiology may be divided into three distinct phases: a) host tissue damage, b) donor T-cells activation, and c) cytokine- and cell-mediated target tissue apoptosis and lysis. Therefore, inflammatory process is a common underlying mechanisms leading to aGVHD. Anti-inflammatory mechanism may constitute an important protection against the development of aGVHD and in this setting protein C (PC) might play a significant role for its cytoprotective effect. PC is activated on the surface of endothelial cells (EC) by the thrombin-thrombomodulin complex, when PC is bound to its receptor, the endothelial protein C receptor (EPCR). Soluble form of EPCR (sEPCR) inhibits PC activation on EC by competing with membrane-associated EPCR. Increased levels of sEPCR are observed during inflammatory process and constitutively are associated with A3 haplotype of EPCR gene. In patients with haplotype A3 of EPCR gene the elevated sEPCR levels are due to increased EPCR shedding by metalloprotease ADAM17 and to the alternative mRNA splicing in A3-carrying cells. The aim of the present study was to investigate if haplotype A3 of EPCR gene is associated with aGVHD development. We studied EPCR gene haplotypes (A1, A2, and A3) in 60 consecutive patients underwent allogeneic HSCT and evaluated their correlation with aGVHD onset. Of the 60 patients enrolled in the study, 27 (45%) developed an aGVHD (14 skin, 5 GI, 2 liver, 2 skin+liver, 2 skin+GI+liver, 1 skin+GI, and 1 GI+liver). Sixteen cases out of 60 (27%) showed A3 haplotype (both in homozygosis and heterozygosis) and in this subgroup of patients 7 out of 16 showed an aGVHD (44%). In 44 patient out of 60 with non-A3 haplotype (73% of cases), aGVHD was observed in 20 of them (45%). In the present study we didn't find any significant correlation between the A3 haplotype of EPCR gene and the incidence of aGVHD. This lack of correlation could be partially due to the reduced number of cases studied and to the presence of many heterozygosis patients for A3 haplotype. However, the evaluation of sEPCR levels in the patients studied for EPCR gene haplotypes is in progress for a more precise analysis of the results.

1650

HAEMATOPOIETIC STEM CELL TRANSPLANT AS A FEASIBLE CONSOLIDATION POST SALVAGE THERAPY FOR FAVOURABLE CYTOGENETICS ACUTE MYELOID LEUKAEMIA IN SECOND COMPLETE REMISSION: A SINGLE CENTRE EXPERIENCE

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Background. Acute Myeloid Leukaemia (AML) with favourable cytogenetics are usually treated with success with just chemotherapy. However, relapsed patients usually still do well after salvage therapy and post salvage haematopoietic stem cell transplantation (HSCT). Autologous HCST is a feasible option and is effective in this group of patients and is able to give long term remission. *Aims.* We wanted to examine the characteristics and overall survival of patients with AML with favourable cytogenetics who had a HSCT. *Methods.* We examine retrospectively our centre's experience in AML who underwent HSCT from the year 1999 to January 2010. Patients who were transplanted and had favourable cytogenetics results on diagnosis were included and analysed for demographics, disease details, remission status and overall survival. Acute promyelocytic leukaemia (APML) were induced and consolidated with All-trans retinoic acid (ATRA) and idarubicin. Relapsed patients were salvaged with arsenic trioxide with or without chemotherapy(high dose cytarabine). AML with t(8;21) were induced with 2 cycles of 3+7 regimen(daunorubicin with cytarabine) and consolidated with 2 to 3 cycles of high dose cytarabine with or without mitoxandrone or idarubicin. Relapsed patients were salvaged with Fludarabine+cytarabine+GCSF+Idarubicin(FLAG-Ida). Both sets of patients had peripheral blood stem cells collected with Etoposide/GCSF and had myeloablative conditioning regimen for the HSCT(busulfan and cyclophosphamide). *Results.* We had a total of 133 AML patients transplanted in our centre in the period with 14.3%(19) with favourable cytogenetics. 9.8%(13) had t(15;17) and 4.5%(6) had t(8;21). There was significant difference(P=0.04) in 4-year overall survival among those with(79%) and without(49%) favourable cytogenetics. Of the 13 patient with t(15;17), 1 had been transplanted in first complete remission(CR1) whereas 12 had HSCT in second complete remission(CR2). The only patient transplanted in CR1 had an autologous HSCT and died due to failure of engraftment. Of the remaining 12 patients transplanted in CR2, 2 had allogenic HSCT and 10 had autologous HSCT. Of the 6 patient with t(8;21), 2 had autologous HSCT in CR1, 1 had autologous HSCT in CR2, 1 had allogenic HSCT in CR1, 1 had allogenic HSCT in CR2 and 1 had allogenic HSCT in relapsed refractory disease. All patients were still alive on assessment except for the patient who had relapsed refractory disease who died 3 months post transplant due to relapse. *Conclusions.* AML with favourable cytogenetics could be salvaged with chemotherapy and transplanted in CR2 with good overall survival. Both autologous and allogenic HSCT are feasible options.

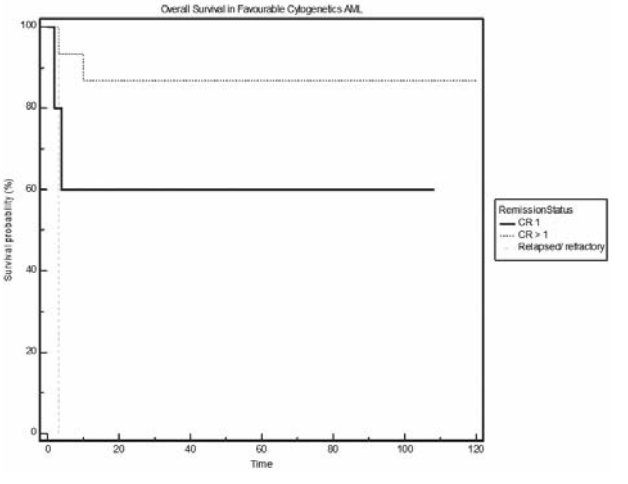

Figure. Remission status and survival in favourable AML.

1651

MAGIC-TT: A NEW STRATEGY OF STEM CELL TRANSPLANTATION TO PROMOTE DONOR CELLS ENGRAFTMENT IN BONE MARROW AND HEMATOPOIETIC RECONSTITUTION

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Background. It is tempting and challenging to facilitate hematopoietic stem cells (HSCs) homing to bone marrow. If it is successful, we can easily perform umbilical cord blood transplantation for adults, resulting in better engraftment with less donor cells, as well as less graft-versus-host-disease (GVHD) in allogenic transplantation. It is a big problem that few HSCs (some reported as 1.56%±0.32%) can homing back to bone marrow when infused from veins, some researchers therefore, tried to infuse HSCs from artery or directly into bone marrow. Unfortunately, improvement was limited. *Aims.* To establish a novel stem cell transplantation strategy named MagIC-TT (Magnetism-induced cell targeting transplantation) which can facilitates donor cells engraftment and hematopoietic reconstitution. *Methods.* Male FVB-GFP (H-2p) transgenic mice were used as donors, and female BALB/C (H-2d) mice as recipients. In this (H-2p→H-2d) allogenic bone marrow transplantation model, great immune barrier has to be overcome. At first, 4 groups of mice (n=10) were preconditioned with 8 Gy total body irra-

diation, and then each mouse was infused with 5×10e6 GFP+ bone marrow nucleus cells from vena caudalis or from the right femur bone, with or without MagIC-TT method (named as MagIC-TT i.v group, MagIC-TT f.b group, control i.v group and control f.b group, respectively). Routinely, mice survival, weight, GVHD manifestation and peripheral blood test were done. Also, fluorescence activated cell sorting (FACS), quantitative real-time RT-PCR, magnetic resonance imaging (MRI) and pathological examinations were used to trace the donor GFP cells. Subsequently, serially diluted donor GFP+ bone marrow nucleus cells were used in 5 more MagIC-TT i.v groups (n=10), compared with control groups, to identify the efficiency improved by MagIC-TT method. *Results.* Compared with control groups, much more donor cells were found rapidly homing to bone marrow within 24h in both MagIC-TT groups identified by pathological examinations, FACS and MRI, etc. Hematopoietic reconstitution in both MagIC-TT groups was much faster than control groups. In both MagIC-TT groups, more mice survived on +300d, with less GVHD. Even compared with dead ones, mice in MagIC-TT groups survived longer. The GFP cells distribution in organs of survival mice demonstrated no difference among different groups. The efficiency of donor cells engraftment was markedly improved by MagIC-TT method. *Summary and Conclusions.* 1. The MagIC-TT proves to be an effective and safe strategy, which facilitates donor cells engraftment in bone marrow and hematopoietic reconstitution. 2. The mechanism of this strategy is quite different from traditional transplantation, which means less donor cells but better engraftment. It will be helpful to improve the outcome of HLA mismatch allogenic transplantation, cord blood transplantation to adults, and GVHD control. 3. MagIC-TT may also be used in other cell transplantations or cell therapy.

1652

IMPLICATIONS OF TRANSPLANTED DOSES OF VARIOUS PROGENITOR CELLS ON DEVELOPMENT OF CHRONIC GRAFT VERSUS HOST DISEASE IN ALLOGENEIC STEM CELL TRANSPLANTATION OF PEDIATRIC PATIENTS

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Background. Chronic graft-versus-host-disease (cGVHD) remains the most frequent late complication after allogeneic hematopoietic stem cell transplantation (HSCT). It is known that various cellular interactions and responses are involved in the pathogenesis of cGVHD and that the more cells are infused, the incidences of GVHD become higher. However, there are only a few studies which specifically focus on infused cells by cellular composition and their influences on cGHVD, especially in pediatric patients. *Aims.* We analyzed the influences of absolute numbers and relative proportions of various immune competent progenitor cells in peripheral blood (PB) allograft on cGVHD in pediatric patients. *Methods.* We retrospectively analyzed 37 children given HLA-matched allogeneic G-CSF mobilized PB HSCTs for malignant (n=25) or nonmalignant (n=12) diseases. *Results.* The donor was an HLA-identical sibling in 8 cases and an unrelated donor in 29. Conditioning regimens were varied according to the disease status. The GVHD prophylaxis consisted of cyclosporine and prednisolone in sibling donor, tacrolimus and methotrexate in others. Chronic GVHD developed in 7 children (18.9%) with malignant disease at a median of 166 days after HSCT and three-year cGVHD probability was 20.6%. In 37 transplants, the median numbers of infused total nucleated cells (TNCs), $CD34^+$ cells were $20.7(7.9\text{-}218.4)\times10^8$/kg, $12.1(2.2\text{-}113.2)\times10^6$/kg, respectively. As a result of flow cytometric analysis of allograft, the median numbers of infused $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, $CD16^+CD56^+$ cells were $4.1(1.9\text{-}21)\times10^8$/kg, $2.2(1.2\text{-}10.8)\times10^8$/kg, $1.7(0.6\text{-}10.8)\times10^8$/kg, $1.3(0.5\text{-}5.3)\times10^8$/kg, $0.8(0.2\text{-}5.1)\times10^8$/kg, respectively. Stepwise Cox regression analysis revealed that the $CD4^+$ to $CD3^+$ ratio was significantly correlated with the development of cGVHD (HR 1.185; 95% CI 1.042-1.348; P=0.01). The incidence of cGVHD was statistically higher in those receiving allograft with more than 0.53 in $CD4^+$ to $CD3^+$ ratio (P=0.03). And there were increasing trends in cGVHD incidence when $CD4^+$ to $CD8^+$ ratio was over 1.1 (P=0.09), when infused $CD34^+$ cells were higher than 7.43×10^6/kg (P=0.18) and when infused $CD19^+$ cells were higher than 1.3×10^8/kg (P=0.16), although not statistically correlated. Otherwise the associations of transplanted doses of TNC, $CD34^+$, $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$ and $CD16^+56^+$ cells with cGVHD were not statistically significant and there was no trends between cell dose with cGVHD. Also there was no definite correlation between cell numbers or compositions with organs involved or extents of the cGVHD. The overall survival and event free survival rates were 78.3% and 31.9%, respectively. T here were 10 events which composed of 7 relapses, 1 non-relapse-mortality, and 2 secondary malignancies without engraftment failure. *Summary/conclusions.* The incidence of cGVHD was significantly correlated with high $CD4^+$ to $CD3^+$ ratio in allogeneic G-CSF mobilized PB HSCTs of pediatric patients. This result suggests that $CD3^+$ and $CD4^+$ cell may play an important role in development of cGVHD. $CD4^+$ cells could be many lineage progenitors rather than regulatory T cells known as GVHD modulators, so further studies for additional analysis of $CD4^+$ cells and also standardization of T and B cell doses for clinical adjustment to reduce cGVHD with proper engraftment are warranted.

1653

A FATAL MICROASCUS CINEREUS (ANAMORPH SCOPULARIOPSIS) BRAIN ABSCESS IN AN ALLOGENEIC BONE MARROW TRANSPLANT RECIPIENT

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Background. Microascus cinereus, an ascomycetous mold in the order Microascales, family Microascaceae, is one of the most common species of the genus Microascus. It is an uncommon pathogen in humans or animals. *Aims.* We report the second case of brain abscess due to M. cinereus in a bone marrow transplant recipient and discuss the mycological findings. *Methods.* Retrospective analysis of clinical chart and microbiology *Results. Results.* A 34-year-old male allogeneic hematopoietic stem cell transplant (allo-HSCT) recipient for Hodgkin lymphoma was admitted 4 years post-transplantation with right lateral homonym hemianopsia. At the time of this complication, he was treated with tacrolimus, corticosteroids and mycophenolate mofetil for extensive chronic severe graft-versus-host disease (GVHD) he had developed after receiving DLI to treat relapsed Hodgkin lymphoma, as well as ciprofloxacine, metronidazole and voriconazole. A magnetic resonance imaging scan (MRI) of the brain revealed a 1,5- by 2-cm enhancing lesion in the posterior part of the left internal capsule. Liposomal amphotericin B was added to voriconazole, and a stereotactic brain biopsy performed that was unrevealing most likely for technical reasons. Two weeks later, he developed a complete right sensory-motor hemiplegia. A second MRI showed no change in the size of the lesion. The patient underwent a second stereotactic-guided aspiration of the abscess cavity. Direct smear of biopsy specimen prepared with Fungi-Fluor™ showed septate hyphae and oblong cells in short chains (Figure 1). Abscess material from the lesion was inoculated onto potato dextrose, sabouraud's dextrose and brain heart infusion agar with and without chloramphenicol/gentamicin. Growth was first visible on brain heart infusion agar and developed into small grayish mold colonies. Within one week of incubation at 30°C the mold was also isolated on potato dextrose and sabouraud dextrose agar (Figure 2). Microscopic examination of potato dextrose agar slide revealed conidiogenous cells cylindrical or slightly tapering, annellidic, 4 to 5.5 × 2.5 to 3 µm. arising from either single or penicillate flask (Figure 3). These features were consistent with a dematiaceous Scopulariopsis species. Ascomata spherical, black, 130 to 340 µm diameter, with a short-cylindrical ostiolar beak were seen after 2 to 3 weeks of incubation (Figure 4). Partial sequencing of the large ribosomal subunit 28S identified the mold as Microascus cinereus, in accordance with the morphological.

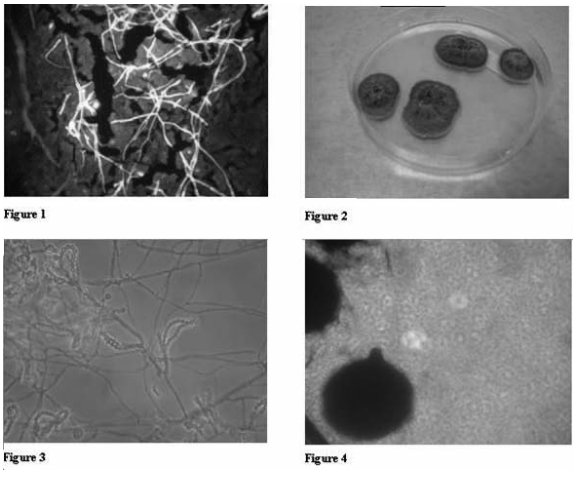

Figure. Morphological features of Microascus cinereus.

Results. Although posaconazole was added to the treatment, the patient died of progressive fungal infection within days. *Conclusions.* We describe the second case of brain abscess caused by M. cinereus after allo-HSCT, an organism that is uncommon and rarely pathogenic in humans. Predisposing factors for infection with this organism included severe immunosuppression, GVHD and exposure to broad-spectrum antibiotics. The isolation of this organism from brain abscess tissue extends the list of known neurotropic dematiaceous organisms capable of causing cerebral phaeohyphomycosis.

1654

PROTECTIVE EFFECT OF DEFIBROTIDE ON THE ENDOTHELIAL ACTIVATION INDUCED BY AUTOLOGOUS SCT ON BOTH MACROVASCULAR AND MICROVASCULAR LOCATIONS

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There is evidence of endothelial activation and damage associated with autologous hematopoietic stem cell transplantation (SCT). Most of the complications appearing early after HCT have a microvascular location. The activation and damage of endothelial cells of both macro (HUVEC) and microvascular (HMEC-1) origin occurring early during autologous HSCT and the potential protective effect of Defibrotide (DF, at 100 μg/mL) were investigated. Sera samples were collected before conditioning (Pre), at the time of transplantation (day 0), and at days 7, 14 and 21 after SCT. Changes in the expression of endothelial cell receptors at the cell surface, presence and reactivity of extracellular adhesive proteins, and the signalling pathways involved were analyzed. The expression of ICAM-1 at the cell surface increased progressively in both HUVEC and HMEC-1. However, a more prothrombotic profile was denoted for HMEC-1, especially at the time of transplantation (day 0), which reflects the deleterious effect of the conditioning treatment on the endothelium especially at a microvascular location. Interestingly, this observation correlated with a higher increase in the expression of both tissue factor (TF) and von Willebrand factor (VWF) on the extracellular matrix. Previous exposure and continuous incubation of cells with DF prevented the signs of activation and damage induced by the autologous sera, being more effective in the HMEC-1 setting. Taken together, these observations indicate that DF has a potential protective effect on the endothelium at both macrovascular and microvascular locations.

1655

POST-TRANSPLANT CHIMERISM ANALYSES USING DNA EXTRACTED FROM ASPIRATE PARTICLE SMEARS BETTER PREDICTS DISEASE RELAPSE THAN FROM CONVENTIONAL ASPIRATE SAMPLES IN PEDIATRIC PATIENTS WITH AML

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Background. Chimerism analyses using short tandem repeat (STR) markers has become an essential workup for post-transplant surveillance of engraftment and disease relapse. Conventionally, DNA from bone marrow aspirate drawn into EDTA bottles (Conv-BM) is used for chimerism analyses. However, it is conceivable that recipient DNA (%R) from Conv-BM might be underestimated due to potential peripheral blood dilution. In this regard, DNA from aspirate particle smears (PS-BM) would best represent the chimerism status of BM. *Aims.* This study was performed to compare chimerism status from Conv-BM with those from PS-BM in a series of pediatric AML. *Materials and methods.* The study subjects were pediatric patients with AML who experienced disease relapse after allogeneic HSCT. Chimerism status was routinely monitored by quantitative analyses of informative STR markers using the PCR-gene scan technique. DNA samples were additionally obtained from PS-BM at 2 time-points in selected cases, immediately prior to relapse (Pre-Rel) and at the time of relapse (Rel). Chimerism analyses were performed on the same set of informative STR as previously selected for Conv-BM. The %R obtained from PS-BM were compared with those from Conv-BM. *Results.* Total 7 cases of AML were selected in a consecutive manner (5 boys and 2 girls; age range 2-17 years, median 13 years). In all patients except for Patient 5, %R from Conv-BM was 0% at Pre-Rel (complete chimerism). On the other hand, chimerism analyses using DNA from PS-BM revealed mixed chimerism in all cases, with %R from 1.1% up to 8% (mean 3.7%). Also at the time of relapse, a significant difference was observed between %R from Conv-BM and from PS-BM, with Δ%R (%R[Conv-BM] - %R[PS-BM]) from -34.5% to 9.6% (mean -7.5%). *Conclusion.* Chimerism analyses using DNA obtained from particle smears better predicted disease relapse than from conventional aspirate samples in pediatric AML. Potential underestimation of disease markers due to dilution effect need to be addressed in other diagnostic workup in hematologic malignancy.

1656

IMMUNE RECONSTITUTION AT DAY +100 - AN IMPORTANT DETERMINANT OF COMPLICATIONS FOLLOWING HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. The immune recovery correlates with the outcome after hematopoietic stem cell transplantation (HSCT). Different lymphocytes subsets at various time points after HSCT were evaluated. *Aim.* The aim of present study was to evaluate the impact of lymphocyte subset reconstitution on relapse rate and opportunistic infectious complications following HSCT. The group of 56 patients, female to male 25/31, with median age 40 (range 19-65), underwent allogeneic (27 pts) or autologous (29 pts) stem cell transplantation due to hematologic malignancies, were enrolled in this study. *Methods.* T lymphocyte subsets (T CD3, CD4, CD8, TCR gamma/delta, Treg and NKT) and NK cells were enumerated in fresh whole blood EDTA samples by direct 3 or 4-colour immunofluorescence analysis (FACScalibur, Becton Dickinson) at day 100 after transplant. The controls were blood samples obtained from healthy donors. *Results.* We have noticed that immune recovery after alloHSCT differs from that after autoHSCT. Counts of NKT cells were higher after autoHSCT in comparison with controls (P=0.02) and Treg (P=0.06) and NK cells (P=0.0003) were lower, whereas in alloHSCT group these subsets of lymphocytes were similar to controls. Only number of CD4 T cells were significantly lower in both groups of transplants in comparison with the control (P=0.0001). We observed the higher relapse rate in patients (n=22)with lower counts at day +100 of: CD4 T-cells (P=0.0001), Treg (P=0.0001) and NK cells (P=0.0002). CMV reactivation (n=13) was strongly correlated with low number of CD3 (P=0.0497), CD4 (P=0.0001) and Treg (P=0.0153) subset. Besides in patients with extremely low counts of CD3, CD4 and Treg measured at day +100 extrapulmonary tuberculosis (n=2) and invasive fungal infections (n=2) developed subsequently. *Conclusion.* The count of lymphocyte subsets (CD3, CD4, Treg and NK) measured at day 100 seems to be associated with the risk of opportunistic infectious complications and relapses in patients after HSCT. Due to heterogeneity and small sample size our results should be determined in a larger study.

1657

MOBILIZATION OF PERIPHERAL BLOOD STEM CELLS (PBSC) FOR AUTOLOGOUS STEM CELLTRANSPLANTATION (ASCT) IN PATIENTS WITH LYMPHOMA AND HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTION

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Background and Aim. Several studies have demonstrated the feasibility of ASCT in patients with lymphoma and HIV infection. In addition to advanced age, radiotherapy, bone marrow involvement and previous myelosuppressive therapy with alkylating agents or purine analogues, HIV infection has been considered a risk factor for poor mobilization. The aim of this study was to evaluate the results of the mobilization schedules of PBSC in patients with lymphoma and HIV infection. *Methods.* Retrospective study of patients with lymphoma and HIV infection, who have received an ASCT in seven Spanish hospitals. Demographic, clinical, biological data, previous chemotherapy and outcome were collected. Mobilization schedules were classified in two groups: G-CSF and G-CSF^{+} chemotherapy (CHT). *Results.* Thirty-four patients were

included, with mean (SD)age 44 (9) yr., 31 (91%) were male, and all received highly active antiretroviral therapy (HAART). Twenty-two patients (65%) were diagnosed of non-Hodgkin lymphoma (NHL), and 12 (35%) of Hodgkin lymphoma (HL). The stage of the lymphoma was limited (I-II) in 7 patients (21%), and advanced (III-IV) in 27 (79%). Twenty-one patients (65%) presented extranodal involvement and 14 (41%) showed an International Prognosis Index (IPI)>2. The number of CHT regimens received prior to ASCT was of 1 in 5 patients (15%), 2 in 25 (73%) and 3 in 4 (12%). Disease status before ASCT was first remission (CR1) in 12 patients (35%), second or further remission (CR>1) in 11 (32%), and partial response (PR) in 11 (32%). Patients mobilized with G-CSF (n=20) were comparable with those mobilized with G-CSF+CHT (n=14) for age, type of lymphoma, type of response at ASCT and number of prior CHT regimens. Sixteen out of 20 patients (80%) mobilized successfully wit G-CSF regimen, and 11 out of 14 (78%) mobilized with G-CSF+CHT. The number of $CD34^+$ cells was higher in patients successfully mobilized with G-CSF^+CHT than in those with G-CSF (mean [SD] 8.7 [7.7] $\times 10^6$ CD34 cells/kg *vs.* 3.9 [1.3] x106 CD34 cells/kg, P=0.459). Disease status pre-ASCT was the only predictive factor for successful mobilization (20/22 patients [91%] in CR *vs.* 5/12 [42%] in PR, P=0.038). No differences were observed in mobilization between patients in CR1 and CR>1. Neither age nor type of lymphoma, stage of disease, LDH level or performance status prior to mobilization had influence on the success of mobilization. No differences were observed in neutrophil and platelet engraftment between patients mobilized with G-CSF or G-CSF^+CHT. *Conclusions.* In HIV-related lymphomas the rate of successful PBSC mobilization was similar with G-CSF or G-CSF^+CHT. The only risk factor for successful mobilization was the disease status prior to ASCT.

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1658

PROGNOSTIC INFLUENCE OF ELEVATED PRETRANSPLANT SERUM FERRITIN ON THE RESULTS AFTER ALLOGENIC HEMATOPOETIC STEM CELL TRANSPLANTATION

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Background. Iron overload is common in patients undergoing hematopoetic stem cell transplantation. Several studies have reported that an elevated pretransplantation serum ferritin was associated with lower overall survival and increased nonrelapse mortality. Iron overload increases the risk of infection, veno-occlusive disease and might influence the risk of acute and chronic GvHD. *Patients and methods.* We evaluated 86 patients (pts) retrospectively who were known the level of serum ferritin before the beginning of the conditioning procedure. These patients underwent allogenic hematopoetic stem cell transplantation (HSCT) between December 1998 and November 2009. The group included 22 pts with acute myeloid leukemia, 6 pts with acute lymphoid leukemia, 6 pts with chronic myeloid leukemia, 13 pts with myelodysplastic syndrome, 6 pts with primary myelofibrosis, 9 pts with aplastic anemia, 22 pts with non- Hodgkin's lymphoma and 2 pts with other disease. The median age was 50 years (range, 19-66 years). There were 53 males and 35 females. Fifty seven patients (67%) received a myeloablative conditioning, whereas a reduced intensity conditioning was administered to 28 patients (33%). Most patients (97%) received peripheral blood stem cells as a graft. *Results.* The median level of serum ferritin was 820 ug/L (range, 2-10 466 ug/L). Median follow-up period after HSCT was 11 months (range, 1-144 months). The patients were divided into two groups (group with low ferritin <500 ug/L and group with high ferritin >500 ug/). At 5 years patients in the group with high ferritin had a significantly inferior overal survival than those in the low ferritin group (72% *vs.* 38%, P<0,001). Patients in the high ferritin group more likely to die of infection (in four cases was occured aspergilosis). We have shown a slight diference in relapse rate (13% *vs.* 29%). There was no statistical difference in the cumulative incidence of GvHD. The time to engraftment was shorter in the first group (median 15 days, range: 8 - 44 days) compared with the high ferritin group (median 16 days, range 10 - 30 days). *Conclusion.* Our findings indicate that patients with elevated pretransplantation serum ferritin level had inferior survival because of increased non-relapse mortality, mainly from infection. Iron overload may adversely affect the time to engraftment.

1659

PREDICTIVE VALUE OF SCORING SYSTEM WITH AUTOANTIBODY EXPRESSIONS IN ALLOGENEIC STEM CELL RECIPIENTS

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Background. In our previous study, patients expressing autoantibodies especially ANA showed a better survival in allogeneic stem cell recipients. *Aims.* The current study was designed to implicate the expression of autoantibodies as an prognostic marker after allogeneic stem cell transplantation (SCT). *Methods.* A total of 144 consecutive patients who underwent allogeneic SCT from November 2001 to Sep 2009 and survived at least 3 months were included in the current study. ANA and anti-dsDNA were screened at 3, 6, 12 month and yearly thereafter. *Results.* ANA was positive in 31 patients (21.5%) at median 364 days (range, 90-1141) and anti-dsDNA (>3.0 Iu/mL) in 76 (52.8%) at median 100 days (range, 46-1458). In the multivariate analysis, ANA [hazard ratio (HR) 0.315] and anti-dsDNA >3.0 Iu/mL (HR 0.328) were identified as good prognostic factors for survival and relapse. When scored as the number of autoantibody expression with ANA and anti-dsDNA; score 0 as no autoantibody expression, score 1 as one and score 2 as two, the patients for score 2 showed a 91.8% 5-yr overall survival (OS) (HR 0.075, P<0.001) and 0.4% relapse rate (HR 0.059, P<0.006), those for score 1 a 64.1% 5-yr OS (HR 0.352, P<0.001) and 26.3% relapse rate (HR 0.422, P=0.011), and those for score 0 a 35.7% 5-yr OS and 53.7% relapse rate. *Conclusion.* Patients expressing multiple autoantibodies (ANA and anti-dsDNA) showed a better survival and lower relapse rate in allogeneic stem cell transplantation settings. Scoring with autoantibody expressions could be used as a prognostic marker for long-term survival and lower relapse risk after allogeneic SCT.

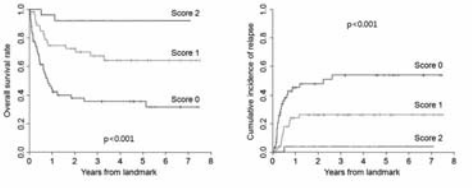

Figure. OS and relapse according to the autoantibody score.

1660

COUNTING OF PERIPHERAL BLOOD STEM CELL: CD34 BY FLOW CYTOMETRY AND HEMATOPOIETIC PROGENITOR CELL ON SYSMEX® XE-2100 AFTER MODIFICATION OF SOFTWARE AND REAGENTS XE-HPC MASTER

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During the organization of collection of PBSC, a crucial problem is to determine the best time to start the harvest. Circulating hematopoietic stem cells (CHSC) evaluation is based on counting of CD34+ cells by IF. This method is expensive, complex, time consuming and induces organization difficulties as regard to bed occupancy and staff timetable. With the increasing acceptance of stem cell transplantation as a standard therapy worldwide, there has been an accompanying increase in demand for technologies that can detect and count progenitor cells. An automatic counter, the Sysmex XE 2100 is equipped with a unique Immature Myeloid Information (IMI) channel, a specially designed unit for the detection of immature myeloid cells which are resistant to lysis reagent and HPC are counted on the basis of direct current and radiofrequency in the IMI-HPC area. The HPC parameter of the IMI channel is used for screening the presence of haematopoietic progenitor (stem) cells in peripheral blood and cord blood samples. Sysmex has developed a hematology analyzer that identifies a small population of immature myeloid cells in the peripheral blood according to cell size, cell density and differential lysis resistance. Since 2004, Sysmex Company undertook software and reagents modification of this cellular counting. The goal of this study was to determine the correlation between the two methods of counting: HPC and CD34 by IF, on peripheral blood sample. We have compared also the results of HPC count on Sysmex XE-2100 and $CD34^+$ cells count before and after software and reagents

modification of HPC counting. We have analyzed HPC and CD34 counting for 95 and 67 CHSC samples before and after modified XE-HPC master. Our results showed a significant correlation between the quantification of the CD34 by IF and HPC before modification (R^2=0.89), this correlation coefficient was not more than 0.53 after the modification. The analysis of these results enabled us to propose a minimal threshold from 20 HPC/mm^3, value of lower part of which, a numeration of the CD34 was required before the modification (R^2=0.87), this is not true after software and reagents modification (R^2 =0.46). The interest of the HPC count is that it is time and cost saving, and thus can be used as a first time method before harvesting stem cells, but, regrettably, the modifications of reagent and software seem to have been out of favor with this promising methodology.

1661

STUDY ON THE IMMUNO-SUPPRESSIVE CHARACTERISTICS OF HUMAN DENTAL PULP DERIVED MESENCHYMAL STEM CELLS ON T CELLS IN-VITRO: INITIAL STUDY OUTPUTS

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Recent studies demonstrated that bone marrow (BM-) derived mesenchymal stem cells (MCS) have immuno-suppressive and/or immunomodulator characteristics on T-cells and can be used for the purpose of inhibition and/or treatment of many immunodeficiencies (e.g. systemic sclerosis, systemic lupus erythematosus, rheumatoid arthritis and immune cytopenia) particularly multiple sclerosis (MS), and graft *vs.* host disease (GVHD)). In our study, we aimed to demonstrate immunosuppressive effects on in-vitro induced T-cells of human embedded molar dental pulp derived (hDP-) BM-MCS's which show similar characteristics with BM-MCS's in terms of immunophenotypic, structural, proliferation and differentiation capabilities, by the use of miscellaneous experimental instruments.

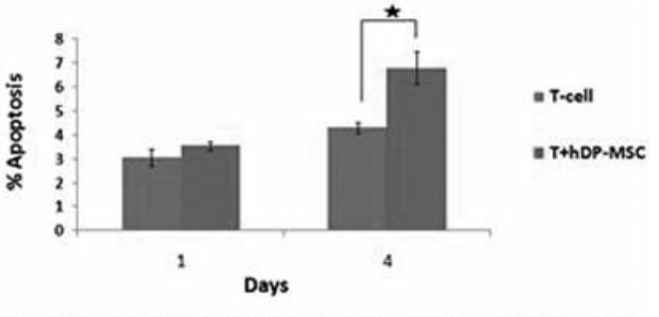

Figure 1: As a result of 4-day indirect co-culture experiments in vitro on hDP-MCS's and T-cells induced by phytohaemagglutinin, we found that hDP-MCS's produced apoptotic effect on T-cells at the 4th day (p<0,05).

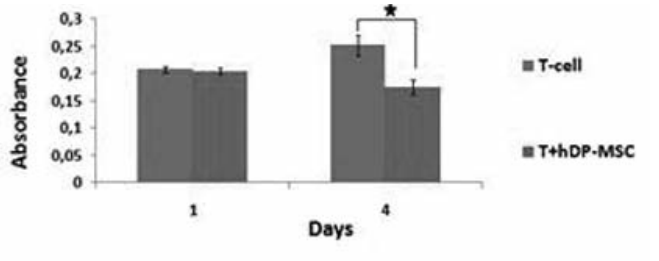

Figure 2: As a result of 4-day indirect co-culture experiments in vitro on hDP-MCS's and T-cells induced by phytohaemagglutinin, we found that hDP-MCS's produced anti-proliferative effect on T-cells at the 4th day (p<0,05).

Figure 1, 2.

For this purpose, MCS's isolated from human embedded dental pulp by enzymatic digestion method were used. For immunophenotypic characterization studies, in flow cytometry device these cells produced positive reaction for CD13, CD44, CD90, CD146 and CD166; reaction was negative for CD3, CD8, CD11b, CD14, CD15, CD19, CD33, CD34, CD45, CD117 and HLA-DR. Telomerase enzyme activity determination, proliferation capacities (by MTT), embryonic gene expressions (Oct4, Rex-1, FoxD3, Sox-2 and Nanog), differentiation studies (osteogenic, chondrogenic, adipogenic, myogenic and neurogenic) and characterization studies were completed. In the second phase, T cells were obtained from peripheral blood samples of healthy adults by the method of negative selection (with rosette-sep kit) and characterization process was completed with flow cytometry, immunohistochemical and gene expression studies. T-cells of which characterization process was completed were induced by phytohaemagglutinin (PHA). In order to study the immunosuppressive effects of MCS's, induced T-cells were taken to indirect coculture system with MCS's in passage 3. For this purpose, MCS's (4×10^5) were transplanted to the bottom of 6-well plates and 2 hour waiting period is left for their adherence. 0.4 micron diameter transwell inserts (main-section) were placed to the wells and 4×10^5 T-cell was placed in each well (at the ratio 1:1) and taken to in-vitro indirect coculture for a period of 4 days. Following each period of time, activity/proliferation capacity in T-cells on the upper side of the intermediate section was measured on spectrophotometer device via MTT method and by evaluating the apoptosis levels after Annexin V-PI staining on flow cytometry device (each experiment was repeated 3 times). After 4-day indirect coculture experiments on hDP-MCS's and induced T cells, we found that hDP-MCS's inhibit proliferation on T cells (P<0,05) and induce apoptosis (P<0,05) (Figure 1, Figure 2). Consequently, initial outputs of our study demonstrated that hDP-MCS's create immuno-suppressive effects on in-vitro induced T-cells. This study was implemented within the scope of project no 108S291 supported by Tubitak.

1662

ALLOGENEIC HEMATOPOIETIC CELL TRANSPLANT CAN AMELIORATE THE ADVERSE IMPACT OF FLT3-ITD MUTATION IN PATIENTS WITH ACUTE MYELOID LEUKEMIA WITH NORMAL CYTOGENETICS

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Background. Acute myeloid leukemia (AML) with normal cytogenetics has historically been regarded as intermediate risk on the basis of karyotypic classification. However, data over the last few years has demonstrated that the presence of recurrent molecular mutations such as the FLT3 internal tandem duplication (FLT3-ITD) is associated with adverse outcomes with high relapse rates and reduced overall survival (OS). The ability of allogeneic transplant to improve outcomes has been demonstrated in some studies but not others. *Aims.* We undertook an analysis of the outcomes of adult patients with AML in our center to determine the impact of FLT3 ITD on outcomes. We assessed whether complete response rate (CR), relapse rate, OS and cumulative probability of relapse following chemotherapy, autologous or allogeneic transplant are affected by the presence of a FLT3-ITD mutation. We also sought to assess if an allograft would be able to ameliorate the adverse impact of the FLT3 mutation amongst our patients. *Methods.* This is a retrospective analysis of adult AML patients with normal cytogenetics treated at our centre between 2003 and 2008. In our center, all patients with AML undergo standard induction with idarubicin for 3 days and cytarabine for 7 days, followed by a similar cycle of consolidation. Patients with normal cytogenetics below age 50 years who have a sibling donor are usually offered an allogeneic transplant. Those who do not, either undergo consolidation with high dose cytarabine or an autologous hematopoietic cell transplant (HCT). Patients with poor risk features such as needing more than one cycle to achieve CR or persistent blasts are considered for an unrelated donor allograft if a suitable donor is found. The conditioning regimen was either myeloablative (busulfan 16 mg/kg and cyclophosphamide 120 mg/kg) or reduced intenstity (fludarabine 120 mg/m^2 and busulphan 8mg/kg to 16mg/kg). Choice of reduced intensity regimen was based on patient's age (above 50 years) or co-morbidities. *Results.* The median duration of follow up was 22 months (0-78 months). Fifty-three patients with median age 51 years (15-61 years) were treated. Of these, 20 underwent autologous HCT, 9 underwent sibling allograft and 6 unrelated donor HCT while the rest received chemotherapy alone. The FLT3-ITD was present in 23 patients. Comparing patients with and without FLT3 mutation, the likelihood of CR after induction was similar (74.1% *vs.* 73.1%) and the probability of OS was 20.9% *vs.* 31.9% (P=0.85). However, the probability of OS among FLT3 positive patients was significantly different based on treatment type: sibling allograft 66.7% *vs.* unrelated donor 20.8% *vs.* autologous allograft 13.8% *vs.* chemotherapy 0% (P<0.001). The cumulative probability of relapse: sibling allograft 33% *vs.* autograft 77% *vs.* unrelated donor 75% (P<0.001). *Conclusions.* The presence of FLT3-ITD confers a poor prognosis on patients with AML with normal

cytogenetics. Our data demonstrates that autologous HCT should not be performed in patients with FLT3-ITD but a sibling allograft may ameliorate the adverse impact of FLT3-ITD mutation.

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TACROLIMUS AND SHORT COURSE SIROLIMUS GRAFT VERSUS HOST DISEASE PROHYLAXIS REGIMEN FOR HLA IDENTICAL SIBLING MYELOABLATIVE PERIHERAL BLOOD STEM CELL TRANSPLANTATION

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Based on the GVHD prevention regimen published by the Dana Farber group we truncated the adminsitration of sirolimus at day 30 post-transplant and maintained tacrolimus until 180 days. The purpose of our study to find a good balance between toxicity and efficacy and graft *vs.* tumor effect with tacrolimus and short sirolimus GVHD profilaxis regimen. From January 2006 to December 2009 we transplanted 46 patients (23 male, 23 female) patients with acute myeloid leukemia(30), acute lymphoid leukemia (6),advannced chronic myeloid leukemia (4), and myelodysplastic syndrome (5). 22/46 patients belonged to the standard risk group and 24/46 to the high risk group. The median age of the patients was 40 (15-61) years. All patients received myeloablative conditioning regimen and unmanipulated peripheral stem cell graft from a HLA indentical sibling donor. The median follow up period is 11.9 (1.2-47) months. All patients engrafted, and reached full donor chimerism and complete remission by bone marrow histology at day 30. The cumulative incidence of acute GVHD was 34%, 13 patients had grade I-II, 2 patients grade III and all of them were steroid responsive. 18/46 patients had chronic GVHD (9 limited, 9 extensive). The toxicity was mild, 3 patients had sinusoidal obstructive syndrome (SOS), 4 patients transplant associated microangiopathy (TMA). We observed 1 CMV diesease, 1 invasive fungal infection, and 8 bacterial blood stream infection. Eleven patients died (5 relapse, 5 chr. GVHD+infection, 1 secondary malignancy). 44/46 (95%) of the patients lived at day 100, 39/46 (85%) at one year, 35/46 (76%) at two year. The probability of four year survival is 63% for the whole cohort, 87% for the standard risk group and 49% for the high risk group of patient. Our patient cohort is in line with the data published by the Dana Farber group but seems to be less toxic regarding SOS syndrome and TMA.

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BASELINE PLATELET COUNT AS A PREDICTOR OF AUTOLOGOUS PERIPHERAL BLOOD PROGENITOR CELL COLLECTION

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Background. It is still a current problem how to predict the best timing to collect stem cell progenitors after chemotherapy, and if the amount collected will allow one or even two transplants. *Aims.* The primary endpoint was to evaluate the CD34 cells/Kg collected. We studied the correlation with basal platelets count, basal haemoglobin and basal WBC count, before mobilization treatment and first stem cell apheresis. *Material and methods.* Progenitor cell collection procedures were retrospectively evaluated in a period of 10 years (2000-2010) to analyze factors predictive of autologous peripheral progenitor blood cell collection. All the patients had a lymphoproliferative disease (68 lymphoma and 47 Multiple Myeloma). 90% of patients received a mobilization schema with chemotherapy and G-CSF. We studied the correlation with basal platelet count, basal haemoglobin and basal WBC count, before mobilization treatment and the first stem cell apheresis. Statistical analysis was made with the SPSS 15.0 for Windows XP program. *Results.* We analyzed a total of 115 progenitors collection procedures in 106 patients (69 male, 37 female; age: median = 56 years; range = 14-73; weight : median =72 Kg; range= 36-110). The median baseline platelet count was 220×10^9/L (range 36-686×10^9/L); the median baseline platelet count at the first apheresis was 123.5×10^9/L (range 4-151×10^9/L); the median total CD34$^+$/Kg collected was 2.8×10^6/Kg (range 0.3-20×10^6/Kg) and the median baseline CD34$^+$/mL in peripheral blood at the first apheresis was 19.890/mL (range 130-510.000); finally the median of CD34$^+$ cells/Kg in the first apheresis was 1.55×10^6/Kg (range 0.2-20×10^6/Kg). Baseline platelet count before mobilization treatment significantly correlated with total CD34$^+$/Kg cell yield and CD34$^+$/Kg cell count in the first apheresis (Spearman r = 0.321, P=0.001 and Spearman R=0.426 P<0.001 respectively). In addition an inverse correlation with the number of apheresis procedures was observed (Spearman r = - 0.277 P=0.003). A direct correlation between the basal platelet count before the first apheresis and total CD34$^+$/Kg cell count (Spearman r = 0.302 P=0.001) was found. *Conclusions.* Baseline platelet count before mobilization and harvest correlates with an adequate CD34+ progenitor cells collection number. Baseline platelet count before mobilization procedures could be used to predict a successful collection.

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INCIDENCE OF GRAFT-VERSUS-HOST DISEASE (GVHD) IN PATIENTS WITH ALLOGENEIC PERIPHERAL HEMATOPOIETIC STEM CELL TRANSPLANTATION AFTER A NON-MYELOABLATIVE CONDITIONING

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Background. Graft-versus-host disease (GVHD) is the most common complication of allogeneic hematopoietic cell transplantation (alloHCT) and may affect the transplant outcome. Its incidence is higher when the preparative regimen used is a non-myeloablative and the stem cell's source is peripheral blood after mobilization. *Aims.* To demonstrate the 10 years incidence of GVHD in 2 Mexican transplant centers using peripheral hematopoietic stem cell transplantation (PHSCT) in related donors after non-myeloablative conditioning. *Methods.* Three hundred and four patients with hematological and non-hematological malignancies that underwent PHSCT after non-myeloablative conditioning between March 1996 and July 2008 were included. The age ranged between 1 and 71 years (median of 30.5). One hundred and eighty-four patients were men and 120 women. They received cyclosporine 4mg/kg per day and intramuscular methotrexate 5 mg/ m^2 in days +1, +3, +5 and +11 for GVHD prophylaxis. *Results.* Two hundred seventy-seven (91.1%) patients were successfully engrafted. One hundred and fifty-eight patients (52%) developed acute and/or chronic GVHD. Of this last number of patients, 104 (34.2%) developed acute GVHD, 91 (29.9%) developed chronic GVHD, and 36 (11.8%) developed both. Thirty five patients (11.5%) who developed acute GVHD were grade III or IV, and 34 (11.2%) of chronic GVHD patients presented in the extensive way. GVHD was the cause of dead in 35 patients (11.5%), even when immunosuppressive therapy (high dose steroids and rituximab) was used. Last death was 24 months ago; subsequently, alemtuzumab was included in GVHD treatment. *Summary/Conclusions.* The incidence of acute and chronic GVHD in our patients is lower than the reported in the literature, even though the source of hematopoietic stem cells was peripheral blood. The mortality rate has decreased due to the introduction of new and more aggressive immunosuppressive agents like alemtuzumab.

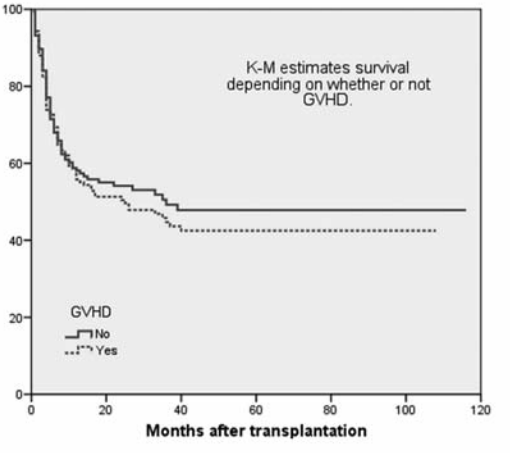

Figure.

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EX VIVO EXPANSION OF UCB HSCS THROUGH COCULTURE WITH OSTEOBLASTS DIFFERENTIATED FROM MESENCHYMAL STEMCELL

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Background. Interaction of HSCs with their specific microenviroment,niche,is critical for maintaining HSCs properties and their fate and one of the most important elements in niche are osteoblasts. *Aim. Ex vivo* expansion of UCB HSCs. *Methods.*After induce difrentiation of MSCs to osteoblasts in 3D,2D condition for ten days we seperated CD34+cells from UCB with MACS and then coculture them with osteoblasts. Result. The mean of $CD34^+$cells in 3D cndition(scaffolds and osteoblasts which diffrentiated from MSCs) was 25/7±4/3 and in 2D conditions in present of osteoblasts was 34/5±3/7 and at the present of cytocines was 64/1±5/7. The mean of Total colonise in 3D cndition(scaffolds and osteoblasts which diffrentiated from MSCs) was 63±3/6 and in 2D conditions in present of osteoblasts was 56±4/2 and at the present of cytocinse was 73±2/1. In Brdu analysis ,Brdu+ cells in 2D condition decreased about 10% and in 3D condition about 8% decreased. *Summary/Conclusions.*;We copare the invitro culture conditions which have the most quiescence without use of expensive cytocinse therfor use of HSCs coculture with osteoblasts can be replaced in use of cytocinse to expansion and maintaining of HSCs properties in selfrenewality.

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ALLERGIC RECONSTITUTION AFTER HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background. In the pathogenesis of allergy, two main types of cells play a role: hematolymphatic cells (mast cells, eosinophils, T cells, B cells) and nonhematolymphatic cells (airway smooth muscle cells, epithelial cells). Hematolymphatic cells, particularly CD4 T cells, appear to play an important role. Immune deficiencies that are caused by defect of hematolyphatic cells are typically cured by allogeneic hematopoietic stem cell transplantation (HSCT). Allogeneic HSCT can also lead to transfer or cure of autoimmune diseases. *Aims.* We aimed to investigate the alteration of allergic tendency in patients undergoing HSCT and to evaluate the relation between allergic reconstitution and graft *vs.* host disease (GVHD) development. *Methods.* Eighteen patients who received HSCT between November 2008 and November 2009 were included. We checked serum total IgE and radioallergosorbent test (RAST) before and after HSCT, and investigated correlation with GVHD. *Results.* Among eighteen patients, two patients underwent autologous HSCT five patients underwent allogenic HSCT from sibling donor, and the others from unrelated donor. Among sixteen patients with allogeneic HSCT, nine patients received peripheral blood, six patients received bone marrow, and one patient received umbilical cord blood. Eleven patients changed their allergic status after HSCT. Six patients increased their total IgE or RAST score, but five patients showed decrease. *Conclusions.* Allergic reconstitution after HSCT shows a tendency towards correlation with source of hematopoietic stem cell rather than GVHD development, probably due to the fact that CD4 T cells play an important role in the pathogenesis of allergy. The most likely mechanism of allergy reconstitution is transfer of either hematopoietic stem cell precursors having a tendency to differentiate into allergy-prone hematolymphatic cell, or mature allergen-specific B or T cells from donor. To determine the likelihoods of transfer of allergy in patients undergoing allogeneic HSCT, large prospective study with inclusion of the allergic status of donor is needed.

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THE TREATMENT OF RESISTANT LIFE-THREATENING INFECTIONS IN PRIMARY IMMUNODEFICIENCIES TREATED WITH BONE MARROW TRANSPLANT IN RELATION TO ALLOGENEIC IMMUNE RECOVERY

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Severe, persistent, non-curable, opportunistic infections are a specific challenge of allogeneic bone marrow transplantation (BMT) in primary immunodeficiencies. The aim of this study was to asses the results of BMT in patients affected by life-treatening, antimicrobial agent-resistant pathogens. In our department 7 allogeneic BMT were performed in 6 children with severe primary immunodeficiencies (4 sibling and 3 matched unrelated donor transplants) since 2007 to 2009. One child underwent BMT twice, because of the graft failure. The group consisted of 5 boys and one girl, aged 6,8 years (range 0,5 - 13). Four children were diagnosed with chronic granulomatous disease (CGD), one child with Omenn syndrome, and another with hyper-IgM syndrome. Ablative conditioning was administered in five and reduced intensity therapy in two cases. The number of transplanted $CD34^+$ cells ranged 2 -8×10^6 per kg (median 4,53×10^6). Leukocyte recovery was observed 9 to 22 days after BMT (median 18 days). Life-threatening, resistant to any antimicrobial treatment infections, present before the transplant procedure, affected four children (57% of transplants). Among them, Staphylococcus epidermidis MRSE MLS was found causing the recurrent sepsis in the child with Omenn syndrome and as the persistent soft tissue infection in the child with CGD. Therapy resistant CMV disease affected two boys: with Omenn syndrome and CGD. Aspergillus flavus caused chronic sinusitis in the girl with CGD. The boy with hiper-IgM syndrome suffered from chronic progressive cholangitis and hepatitis due to Cryptosporidium parvum. In two children with CGD the therapy resistant granulomas in liver and in soft tissue was observed. The therapeutic strategy after BMT for these infections was the application of the antimicrobial drugs together with maintenance of the high serum concentation of immunoglobulins and intensive supportive care until the time of immune recovery and self-ability to eliminate the pathogen. The blood stream MRSE infection was eliminated after leukocyte recovery in the 9th day after BMT. The soft tissue granulomas resolved three weeks after BMT, while normal values of chemiluminescence and NBT tests were found on 28th day. Resistant CMV infection in Omenn child was not detectable in the 78th day, contemporary to T-cell repertoire recovery. Aspergillus sinusitis declined in several weeks after transplant, with the normal granulocyte function score on the day 49th. Cryptosporidium parvum was absent in affected patient in the 131st day, when the immunoglobulin class switching was found sufficient. The granuloma in the liver of one boy with CGD resolved in two months, in the other boy granulomas in his liver decreased during three months after BMT. Until now five children achieved the good physical condition, without symptoms of the primary immunodeficiency. One boy is treated for chronic graft *vs.* host disease. *Conclusions.* 1. The specific feature of transplantation in children with severe primary immunodeficiences were the infections resistant to antimicrobial therapy, found before BMT (4 children, 57%). 2. Antimicrobial agents with the maintenance of high immunoglobulin concentration and proper supportive care until the time of immune recovery was the effective treatment of the severe, resistant, opportunistic infections.

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EFFECTS OF COMBINED APPLICATION RECOMBINANT HUMAN ERYTHROPOIETIN (RHU-EPO) AND GRANULOCYTE COLONY STIMULATING FACTOR (G-CSF) ON HAEMOPOIETIC RECOVERY AFTER HIGH DOSE CHEMOTHERAPY WITH ALLOGENIC STEM CELL TRANSPLANTATION

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From 1994 to 2006 years in Belarussian Research Institute of Haematology 79 patients(42 male, 37 female) with different diagnoses(chronic myeloid leukemia-38, acute myeloid leukemia-10, acute lymphocytic leukemia-7, aplastic anemia-21, multiple myeloma-1, myelofibrosis-1, acute promyelocytic leukemia-1) have been treated by method high dose chemotherapy (HDC) with allogenic stem cell transplantation (SCT).Median age was 30,6 years (range 11-50). All patients have been

divided into 3 groups. The first group (n=33) did not receive any haemopoietic growth factors, the second group (n=34)-received granulocyte colony stimulating factor (G-CSF) (300mkg per day), the third group (n=12) was treated with combination of recombinant human erythropoietin (rHu-EPO) and granulocyte colony stimulating factor (G-CSF). Criteria of haemopoesis restoration after allogenic SCT were following: Hb>90 g/L, erythrocytes > $3,0\times10^{12}$/L leukocytes >$1,0\times10^{9}$/L, neutrofiles>$0,5\times10^{9}$/L, thrombocytes > $20,0\times10^{9}$/L. For comparison of efficiency of haematologic recovery in investigated groups used day of achievement specified above indicators of peripheral blood after allogenic SCT. *Results.* Time of Hb restoration in group of patients received rHu-EPO+G-CSF was 13,8+9,7 *vs.* 20,2+9,8 in group G-CSF (P=0,06) and 22,2+9,1 in group without CSF (P=0,01). Time of erythrocytes restoration in group rHu-EPO+G-CSF was 12,6+9,3 *vs.* 20,8+10,1 in group G-CSF (P=0,017) and 20,8+10,0 in group without CSF (P=0,016). Time of leukocytes restoration in group rHu-EPO+G-CSF was 15,2+5,4 *vs.* 19,6+6,3 in group G-CSF (P=0,03) and 18,8+5,7 in group without CSF (P=0,07). Time of neutrofiles restoration in group received rHu-EPO+G-CSF was 13,3+4,6 *vs.* 19,0+7,3 in group G-CSF (P=0,016) and 19,1+5,5 in group without CSF (P=0,002). Time of thrombocytes restoration didn't have statistical distinctions. *Conclusion.* Time of restoration of Hb, erythrocytes, leukocytes and neutrofiles in group of the patients received rHu-EPO + G-CSF, was both shorter, than in groups G-CSF and with no CSF. This result shows that combined application of rHu-EPO and G-CSF in early posttransplant period is most favorable for the acceleration of restoration of specified above peripheral blood indicators.

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CMV INFECTION AND DISEASE IN PEDIATRIC HEMATOPOIETIC STEM CELL TRANSPLANTATION, EXPERIENCE OF ONE CENTRE

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Background. Cytomegalovirus (CMV) infection remains an important cause of morbidity and mortality in allogeneic hematopoietic stem cell transplantation (Allo-HSCT). Acyclovir prophylaxis, CMV monitoring by RT PCR and preemptive antiviral drugs declined remarkably the incidence of infection and subsequently of disease. However we are still confronted by pejorative prognostic factors that favour CMV infection, recurrence, resistance and disease. *Aims.* To evaluate retrospectively the magnitude of CMV problem and various prognostic factors in one pediatric HSCT centre. *Methods.* In the period between 9/2000 and 6/2007, 81 children having the following recipient (R) / donor (D) pair CMV serostatus: (R^-/D^+) or (R^+/D^-) or (R^+/D^+) were subjected to Allo-HSCT at Debrousse Hospital, Lyon University. All patients received herpes prophylactic acyclovir or native. CMV was monitored by RT-PCR starting from the 1st week post transplant. Ganciclovir (GNC) or Foscavir (FSC) was used mainly as preemptive and curative treatment. *Results.* Out of 81transplants, 40 patients presented CMV infection and 10 CMV disease. The median CMV onset of infection was 39 days post transplant (2 to 68). The mean DNA copies magnitude was 19986(<500-1580000). Of those 40 infected patients, 14 resolved spontaneously and 26 received preemptive antiviral: GNC n=21 (resolution n=16 and resistance n=5) and FSC n= 5 all resisting. Of those resolving CMV DNA copies, 8 patients showed recurrence. The median duration before infection negativity was 125 days (7-625). It shows that 15% of infected patients reached more than 600 days of infection. Six of the 10 diseased patients were cured and the rest died of CMV disease progression. In univariate analysis progressing DNA copies (cDNA) from <500 to positive >500 were responsible of recurrence p0.027, resistance p0.003 and disease p0.007. In multivariate analysis, CMV infection occurred, more significantly with associated (R^+/D^-) pair p0.01, high dose corticosteroids more than 2 mg/kg p0.006, Anti thymocyte globulin (ATG) p0.002 and non bone marrow stem cell source p0.02. Both (R^+/D^-) pair p0.005 and non geno-identical transplant p0.05 were responsible for increased CMV resistance. *Conclusions.* Preemptive treatment should be started at minimal CMV documentation <500 cDNA to prevent recurrence and resistance. CMV positive recipient are better transplanted from positive donor. Prolonged immune suppression should be avoided. Early positive RT PCR CMV in first week post transplant should raise the question about the real source of infection either the donor or recipient.

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SEVERE NEUROTOXICITY ASSOCIATED WITH DIMETHYL SULPHOXIDE FOLLOWING PERIPHERAL BLOOD STEM CELL TRANSPLANTATION

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Dimethyl sulphoxide (DMSO) is used as a cryoprotectant for long-term storage of hematopoietic stem cells (HSC) from the marrow or blood. Various complications during infusion of cryopreserved HSC have been described as nausea, vomiting, transient hypertension or hypotension and anaphylaxis. Until recently, there has been little information on the neurological toxicity of DMSO. DMSO-related neurotoxicity, including transient global amnesia, cerebral infarction, migrene, seizures, encephalopathy and even coma, has been described, mostly as anecdotic events.We are reporting 3 cases of DMSO-related severe, transient neurotoxicity out of 223 autologous peripheral blood stem cell transplantation (PBSCT) performed in our center between 2000.-2009. All patients (pts) - 2 males, 1 female, 51, 59 and 39 years old, have stage III of multiple myeloma. The first-line therapy for all pts was Dexamethasone - based regimen (VAD or D-CEP). A PBSC collection was performed with Cyclophosphamide (4g/m^2) and rhG-CSF (5 μg/kg/day) and optimal numbers of mononuclear cells (MNC- median 8×10^8/kgBW) were harvested. The cells were cryopreserved on 10% DMSO using a controlled-rate freezer and stored at -900C. The final volume cryopreserved was 450-480ml (5-6 bags). After PBSC collection pts underwent autologous PBSCT. The conditioning regimen consisted of Melphalan at a dose 200 mg/m^2. The bags were thawed in a 37o water bath and infused at a rate of 10 mL/min. On day "0" pts were premedicated with Diphenhydramine and Methylprednisolone (1 mg/kgBW). After the infusion of the last bag all pts were developed a complete loss of consciousness accompanied by incontinence, without clonic convulsions or focal neurological sings. Pulse rate and blood pressure were normal. The pts were transferred to intensive care unit (ICU) for ventilation. No laboratory abnormalities, including electrolytes, osmolarity, coagulation screen, serum glucose and enzymes, were evidenced upon repeated testing. Pts were treated with steroids and forced hydration. The urgent CT scan was unremarkable. All pts recovered consciousness in 3-5h after assistance ventilation was started. Finally, they were extubated within 24h and discharged from ICU on day +1. All pts have optimal engraftment.Because of the strict temporal relationship between the infusion and development of neurological sings and their resolution upon forced hydration, we circumstantially attribute the encephalopathy to the infusion of DMSO-contained in the PBSC suspension. The risk-factors for development of DMSO-neurotoxicity are still unclear. The preconditioning exposure to central nervous system (CNS)-penetrating agents and M protein in multiple myeloma pts might contribute to the occurrence of DMSO -associated neurological toxicity, but a large analyzes are needed.

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HIGH-DOSE CYCLOPHOSPHAMIDE, ETOPOSIDE AND BCNU WITH NON-CRYOPRESERVED AUTOLOGOUS HAEMATOPOIETIC STEM CELL TRANSPLANTATION FOR POOR PROGNOSIS HODGKIN'S LYMPHOMA

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Background and aim. Several high dose chemotherapy regimens have been used in autologous transplants for Hodgkin's lymphomas (HL). Most of these high-dose therapy schedules were designed to deliver cytotoxic drugs over a number of days [e.g. 6 days for BEAM, CBV or ICE], freezing of the harvest product was traditionally carried out to maintain cell viability until stem-cell reinfusion. Unfortunately cryopresevation unities are not available in many developing countries so the liquid storage of harvested stem cells, in standard blood refrigerators, is an alternative to cryopreservation. The aim of this study is to demonstrate the feasibility and safety of autotransplants with noncryopreserved peripheral blood stem cells. *Methods.* 10 patients with poor prognosis malignant Hodgkin's lymphoma were treated with high-dose cyclophosphamide (120 mg/kg), etoposide (2100 mg/m^2) and BCNU (400 mg/m^2) followed by reinfusion of autologous non-frozen PSC, which had been stored for 72 hours at 4°C. *Results.* The median time to achieve an absolute neutrophil count greater than 0.5×10^9/L was 13

days (range 11-20 days). The median time to self-sustained platelet count greater than 20×10^9/L was 20 days (range 15-30 days). Eight of the 10 patients are still alive and disease free and one patient died of severe infection. *Conclusion.* We conclude that high-dose chemotherapy with non-frozen autologous bone marrow transplantation is safe in terms of haematopoietic reconstitution and the preliminary follow-up data suggest a useful efficacy. The procedure is easy to perform without requiring costly cryopreservation.

1673

THE APPLICATION OF INTRA-ARTERIAL STEROID IN ACUTE STEROID RESISTANT GASTROINTESTINAL GRAFT VERSUS HOST DISEASE (GVHD)

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Acute graft *vs.* host disease (GVHD) is a major complication of allogeneic bone marrow transplantation and leads to death for most of the patients with resistance to steroid. Application of topical steroid regimens for resistant acute gastrointestinal GVHD allows high doses of steroid to reach the region and overcome resistence which is caused by the decrease of the number and affinity of intestinal steroid receptors. In our clinic, we gave intraarterial steroid infusion treatment of to two patients that we followed for grade 4 acute steroid resistant gastrointestinal GVHD. One of them was a 30 year-old female patient with ALL, and the other was a 34 year-old male patient with AML. Both of the patients were transplanted from full match sibling donors after a preparation regimen with Busulfan and Cyclophosphamide. 3 months after the transplantation, both of the patients developed grade 4 acut gastrointestinal GVHD. And both of them did not respond to 2 mg/kg/day iv prednisolon therapy. The female patient with ALL did not respond to the secondary treatment with ATG and infliximab either. The male with ALL did not have any secondary systemic treatment. 1 mg/kg metilprednisolon infusion to the inferior and superior mesenteric arteries was performed for both of the patients by using angiografi. No complication related to the procedure occurred. And in the following 2 days the frequency of diarrhea decreased and after 3-4 days it dissappeared completely. We believe that the use of intraarterial steroid is easy, safe and efficient for the treatment of acute iv steroid resistent gastrointestinal GVHD.

1674

IMPLICATION OF EARLY LYMPHOCYTE RECOVERY AFTER ALLOGENEIC STEM CELL TRANSPLANTATION IN CHILDREN WITH LEUKEMIA

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Background. The repopulating lymphocytes after allogeneic hematopoietic stem cell transplantation (HSCT) have an important role not only on the prevention of serious infections in the early transplantation period, but on killing the residual leukemic cells by graft-versus-leukemia effect. Previous studies have suggested that earlier lymphocyte recovery after HSCT was associated with a lower relapse and a better survival in adult patients. *Aims.* The aim of this study was to analyze the impact of lymphocyte recovery after HSCT in children with hematologic malignancies. *Methods.* We retrospectively evaluated 69 children transplanted for ALL (n=34), AML (n=26), chronic leukemia (n=7) and juvenile myelomonocytic leukemia (n=2) at the Chonnam National University Hospital. Stem cell sources were: BM (n=46), PBSC (n=10), umbilical cord blood (n = 12), and BM + PBSC (n=1). Matched sibling donor was used in 26, while unrelated donors in 43. The patients were grouped based on absolute lymphocyte count (ALC) <500/μL or ≥500/μL at D+21 and D+30 after transplant: Low at D+21 (n=28) *vs.* High at D+21 (n=41); Low at D+30 (n=19) *vs.* High at D+30 (n=49). The impact of lymphocyte recovery after HSCT on predicting the survival, relapse, transplant-related mortality (TRM), and graft *vs.* host disease (GVHD) was retrospectively analyzed. *Results.* The median age at transplant was 7.1 years (range, 0.4-18.2). Patients with High ALC at D+21 and D+30 had faster neutrophil and platelet engraftment: The median day of neutrophil engraftment (>1,000/μL) was D+16 for High at D+21 *vs.* D+21 for Low at D+21 (P=.001); and D+17 for High at D+30 *vs.* D+20 for Low at D+30 (P=.02), respectively; The median time of platelet engraftment (>20,000/μL) was D+19 for High at D+21 *vs.* D+38 for Low at D+21 (P=.04); and D+22 for High at D+30 *vs.* D+40 for Low at D+30 (P=.07), respectively. The High at D+30 group exhibited a better 5 year overall survival (71% *vs.* 53%, P=.043) and event free survival (72% *vs.* 53%, P =.065) than Low at D+30 group. The incidence of grade II-IV aGVHD and cGVHD was not different by the ALC counts. Also relapse rate was not different between ALC groups. But the Low at D+30 group was associated with a significantly increased risk of TRM (P=.019). In univariate analysis, factors associated with decreased survival were Low ALC at D+30 and high risk in ALL patients, and grade II-IV aGVHD in both ALL and AML. Grade II-IV aGVHD was the only variable with a significantly negative impact on survival by multivariate Cox regression analysis. *Conclusion.* We found that earlier lymphocyte recovery greater than 500/μL on D+30 was associated with faster neutrophil and platelet engraftment, decreased TRM, and better survival without increasing the incidence of GVHD. However, faster lymphocyte recovery was not translated into decreased relapse rate. Serial lymphocyte measurement early posttransplant would be a simple but useful method for predicting transplant outcomes. Further studies incorporating larger number of cases and longer follow-up are warranted in children.

1675

CAN SERUM LACTATE DEHYDROGENASE ENZYME LEVEL BE AN EARLY MARKER OF MYELOID ENGRAFTMENT IN RECIPIENTS OF HEMATOPOIETIC STEM CELL TRANSPLANTATION?

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Background. Lactic Dehydrogenase (LDH) is an intracytoplasmic enzyme, which catalyzes the transformation of pyruvate into lactate and it is reported that serum LDH levels can increase in parallel to the maturation of myeloid progenitors in the bone marrow and the increase of neutorphils in the periferal blood. *Aims.* In the present study, the relationship between myeloid engraftment time and serum LDH enzyme levels in patients, who underwent hematopoietic stem cell transplantation (HSCT) was studied. *Methods.* The whole blood parameters and serum LDH, ALP, GGT, AST and ALT analyses of 25 patients, 24 of whom underwent allogeneic and 1 autologous HSCT were studied thrice weekly. The parameters on the onset of preparation regimen, the day, in which the product infusion was peformed, 5 days before myeloid engraftment, the day of myeloid engraftment and 5 days after engraftment were evaluated and statistically analysed. The study was approved by the local ethics committee. *Results.* Allogeneic HSCT were performed on 25 patients aging between 7 months and 15 years. The AST, ALT and GGT levels of 3 cases were high before transplantation and remained high throughout the transplantation period. The other patients did not have any illness, which could cause high levels of LDH and G-CSF was not used in any patient. No statistically significant difference was present between the LDH levels on the onset of preparation regimen and on the infusion day (median 343 IU/L *vs.* 374 IU/L, P>0,05). It was detected that the LDH levels on 5 days before myeloid engraftment started to increase with statistical significance (median 374 IU/L *vs.* 433 IU/L, P<0.001), and that this increase continued on engraftment day and 5 days after engraftment and that there were no statistically significant difference between the levels on 5 days before engraftment, the day of engraftment and 5 days after engraftment (median 433 IU/L *vs.* 456 IU/L *vs.* 471 IU/L, P>0,05). It was also detected that the results were similar in the evaluation of cases with no elevation of transaminase levels and that LDH enzyme levels were not affected by high levels of transaminases. *Discussion.* LDH is widespread present in many tissues of the body including neutrophils. It is demonstrated that serum LDH levels begin to increase with the increase of neutrophils in patients whom were applied G-CSF because of myeloid aplasia due to chemotherapy and this incerase is shown to originate from neutrophils. But there is no study, which examined the relationship between myeloid engraftment and LDH in patients, whom were applied HSCT. In the present study, it was detected that serum LDH levels begin to increase 5 days before myeloid engraftment It can be suggested that this increase in LDH levels might be related to the increase of myelopoiesis in the bone marrow. In conclusion, the sequential follow up of serum LDH levels in patients undergoing HSCT and the detection of the period of increase in enzyme levels can herald subsequent engraftment.

1676

MOBILIZATION AND HARVESTING OF PERIFERAL BLOOD STEM CELL WITH INTERMEDIATE DOSE ARA-C IN A PREVIOUS POOR MOBILIZER HAEMATOLOGIC MALIGNANCIES PATIENTS

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Background. The inability to mobilize sufficient number of HSC are a significant clinical problem for relapsed/refractory hematologic malignancies patients eligible to receive high-dose chemotherapy and autologous stem cell transplant. Several lines of treatments, use of alchilating agents, cisplatin and fludarabine, are all factors adversely affecting PBPCs mobilitation. *Aims.* In this study we assessed the efficacy and safety of intermediate dose ARA-C given to mobilize PBSCs. *Patients and methods.* Thirty-six patients (23 M; 13 F; median age 61 yrs; range (26-70) with hematologic malignancies: 17 non-Hodgkin's lymphoma (NHL), 1 Hodgkin's Disease (HD), 15 multiple myeloma (MM) and 3 LAM, di cui 1 have failed a previous attempt between January 2007 and May 2009, were scheduled to receive intermediate dose ARA-C to mobilize PBSCs. *Methods.* Patients were primed using intermediate dose of ARA-C administered intravenously at a dose of 800 mg/m^2 every 12 h for 6 consecutive doses, + rhG-CSF 5 or 10 microgr/Kg subcutaneously. *Results.* A median of 3 chemotherapeutic regimens (range 2-7) were previously given and all patients failed prior harvesting of PBSCs. Three patients with MM were given two consecutive autotransplants with Melphalan 200 mg/mq as conditioning regimen. One patient with NHL received 4 cycles of chemotherapy, one patient underwent Bone Marrow Autotransplant and 2 cycles of Ibritumomab Tiuxetan (Zevalin®). Collection of PBSCs was successfull in 34 out of 36 patients (94%) (17 NHL, 1 HD, 13 MM and 3 LAM). Two patients with MM were no mobilizers with intermediate dose of ARA-C also (one of them received two consecutive autologous PBSC while one other showed a refractory disease at the time of mobilization). Harvesting of PBSCs was performed at a median time of 13.2 days (range 7-17 days) after ARA-C administration. The median number of subcutaneous injections of rhG-CSF was 6 (range 4-13). The median number of WBC count was 4200/mmc (range 1400-15000) at the time of collection with CD34$^+$ median number of 1.8 % (range 0.3-6.2). Among the 34 patients who mobilized, in only two cases the required number of CD34$^+$ cells were harvested after two leukapheresis. The median number of CD34 $^+$ cells collected was 7×10^6/Kg (range 2.04-27,7) with 4 as a median number of cryopreserved bags (range 2-8). All patients experienced neutropenia < 500/microL, and 13 out of 34 had febbrile neutropenia (1 to 4 days). Nineteen patients received a median of 1 packed red cell transfusions (range 1-3) and 30 patients a median of 1 apheretic platelet products (range 1-3). No patients experienced WHO grade III-IV mucositis and diarrhoea. *Results.* These data indicate that mobilization and collection of PBSC strongly depend on the type of chemotherapeutic regimen used. Our experience, showed that PBSC collection using intermediate-dose of ARA-C + rhG-CSF is safe and effective in poor mobilize patients with haematologic malignancies. Mobilization and collection of PBPCs were found independent from the number and type of previous chemotherapies. Indeed it is surprising that mobilization was also achieved in the patient with LNH who received several chemotherapies, Bone Marrow Autotransplant and two cycles of Zevalin®.

1677

ALLOGENEIC HAEMATOPOIETIC STEM CELL TRANSPLANTATION (AHSCT) FOR MYELOFIBROSIS RESULTED IN DISAPPEARANCE OF CONSTITUTIONAL SYMPTOMS, REDUCTION OF SPLEEN SIZE AND DECLINE OF THE JAK2V617F ALLELE RATIO

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Background. Allogeneic hematopoietic stem cell transplantation (AHSCT) remains the only curative therapeutic option for primary myelofibrosis (PMF) however its wide use is limited by high rate of transplant-related mortality. We have reported the preliminary results of AHSCT preceded by reduced-intensity conditioning (RIC) for patients with PMF. *Design and methods.* Six patients (3F/3M) with a median age at transplant of 40 years (range 28-51 yrs) were transplanted between 2008 and 2010. The diagnoses were following: PMF (n=4), post-thrombocythemic myelofibrosis (n=1) and post-polycythemic myelofibrosis (n=1). A median spleen size was 18.4 cm (range16-19cm). The constitutional symptoms including cachexia, weakness, weight loss were present in all studied patients. Risk profile according the Lille score was low in 1 patient and intermediate in the remaining 5. 17% of transplanted patients had an intermediate-1 IPSS, 50% -intermediate-2 and 33%- high IPSS. The JAK2V617F mutation analysis was performed using commercially available test MutaScreen (Ipsogen). The JAK2V617 point mutation was detectable in 4 patients. A median JAK2 allele ratio was 26.5% (range 25-90%). *Results.* Before transplant from sibling (4/6) or unrelated donors (2/6), all patients received reduced-intensity conditioning consisting of fludarabine, busulfan and anti-thymocyte globulin. The source of stem cells was as follows: marrow and peripheral blood in 3 patients and only peripheral blood in 3. The median number of transplanted CD34$^+$ cells per kilogram of body weight was 6.03×10^6/L (range 3.7-10.8×10^6/L). The median time to leukocyte engraftment (absolute neutrophil count >0.5×10^9/L) was 18 days (range 15-24 days), and the median time to platelet engraftment (>20×10^9/L) was 25.5 days (range 14-31 days). Two patients eradicated JAK2V617F clone after transplant and 2 patients significantly reduced JAK2 allele levels. The enlarged spleen returned to normal size in all patients within 2 months after transplant together with constitutional symptoms disappearance. Acute graft-versus-host disease was observed in 4 patients and chronic graft-versus-host disease occurred in 3 patients. One patient died 7 months after AHSCT due to infectious complications. The median follow-up after transplant is 7.5 months (range 1-20). *Conclusions.* RIC before AHSCT resulted in high engraftment rate and low transplant-related mortality. This procedure seems to be safe and highly effective.

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THE ROLE OF STEM CELL TRANSPLANTATION FOR PEDIATRIC SOLID TUMORS - SINGLE CENTER EXPERIENCE

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Background. Ewing's sarcoma (ES), neuroblastoma (NB) and germinal cell tumors (GCT) are rare solid tumors in children and adolescents with low survival rates in cases with advanced disease. The role, the type and the indication of high dose chemotherapy, in the complex treatment of these diseases isn't well established. *Aim.* to evaluate retrospectively the impact of high-dose chemotherapy on outcome of pediatric solid tumors patients. *Methods.* 8 patients transplanted in our center between 2003-2009 were included in this analysis: 4 with ES, 3 with NB, 1 with germinal cell tumor. The patients with ES received chemotherapy according to EWS 97 protocol followed by autologous stem cell transplantation (ASCT) with BuMel (Busulfan, melphalan) conditioning in 2 cases, MEC (melphalan, etoposide, carboplatin) conditioning in 1 case and tandem ME (melphalan, etoposide) in one case. The patients with NB received COJEC protocol followed by ASCT with MEC conditioning. All patients received radiotherapy after ASCT. The patient with germinal cell tumor (yolk sac) received 4 courses of BEP (bleomicyn, etoposide, cisplatinum) + 3 courses of ICE (ifosfamide, carbiplatin and etoposide) followed by tandem autologous transplantation with carboplatine - etoposide conditioning. *Results.* the patients with ES were 4 girls with 9, 13, 14 and 18 y diagnosed with ES metastatic disease in 3 cases and localized disease in one case. The graft was obtained after mobilization with ciclofosfamide and G-CSF and contained 3-18×10^6 CD34/Kg. The patients with NB were 2 boys and 1 girl with 4, 5 and 9 y at diagnosis. The graft was obtained after the last course of chemotherapy contained 1,3-5×10^6 CD34/kg. The patient with metastatic germinal cell tumor was a 19 y old boy. The graft obtained after a ICE course contained 7×10^6 CD34/Kg. All patients engrafted after 9-12 days without severe infectious or hemorrhagic complications. The transplant mortality rate (TMR) was 0%. We analyzed the treatment response according to the status of disease at transplantation: localized *vs.* metastatic disease. After ASCT the ES patient with localized disease remained in CR at 5y; 2 patients with ES metastatic disease obtained CR after ASCT, but one relapsed after 9 months; the other ES patient with metatstaic disease never obtained CR, but remained in stable disease 2 y after transplantation. All three NB patients obtained the CR

after ASCT, but relapsed after a median of 12 months. The patient with germinal cell tumor also obtained CR after transplnatation. *Conclusion.* ASCT is a safe procedure, with 0% TMR. The procedure is effective for patients with ES and localized disease before transplantation; in case of NB, the ASCT allows a longer overall survival, without effect on event free survival.

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PLERIXAFOR IS EFFECTIVE AND SAFE FOR STEM CELL MOBILIZATION IN HEAVILY PRETREATED GERM CELL TUMOR PATIENTS

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Background. Up to 10% of germ cell tumor patients require salvage high dose chemotherapy with stem cell support achieving cure rates in the range of 25-60%. Stem cell mobilization may be difficult in these patients due to multiple pretreatments with platin-containing therapies known to seriously hamper stem cell recovery. New strategies to enhance stem cell mobilization are needed for such patients. Recently, Plerixafor has been approved by the FDA and EMEA in combination with G-CSF for stem cell mobilization in patients with multiple myeloma or lymphoma. Plerixafor significantly enhanced the success of the $CD34^+$ cell harvest, even in cases in which prior mobilization attempts have failed. As the mechanism of action of Plerixafor is not disease specific, we utilized Plerixafor off-label in 6 germ cell cancer patients, refractory to G-CSF alone or in combination with chemotherapy for stem cell mobilisation. *Aims.* Assess efficacy of Plerixafor in germ cell tumor patients who were not able to adequately mobilize $CD\ 34^+$ cells for subsequent autologous stem cell transplantation. *Results.* Six germ cell tumor patients provided informed consent and were included in the compassionate use program at three different specialized centers. All patients were heavily pretreated, with a median of 3.33 prior therapies (Δ 1-6). All failed prior mobilization with G-CSF alone or in combination with chemotherapy. Five patients successfully harvested a median of 7.55×10^6 CD34 + cells/kg body weight (Δ 2.0-24.79×106) in a median of 3.6 apheresis days (Δ 2-6). Three patients underwent high dose chemotherapy with autologous stem cell support. Median time to leukocyte engraftment was 10 days (Δ 8-12) Median time to platelet engraftment was 12 days (Δ 12-13). One patient failed platelet engraftment. No Plerixafor related side effects were reported. *Conclusion.* Plerixafor is safe and effective in germ cell tumor patients who have failed prior mobilization therapy. Larger prospective studies are warranted to further assess its application.

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TREATMENT OF SECONDARY PROGRESSIVE MULTIPLE SCLEROSIS WITH AUTOLOGOUS STEM CELL TRANSPLANTATION

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Introduction. High dose chemotherapy followed with autologous stem cell transplantation (ASCT) has been proposed in the last few years as a potential treatment option for patients (pts) with secondary progressive multiple sclerosis (MS). *Aim.* We analyze retrospectively small cohort of pts with secondary progressive MS treated with ASCT within ESTIMS trial. *Methods.* Since 2005. five pts with secondary progressive MS, who have failed to several lines of therapies (Interferon beta, Mitoxantrone...) was treated in our center with ASCT. Median age was 31 (21-52), male/ female 4/1, baseline median disability status scale (EDSS) of 6,0 (3,5-9,0). All of them were mobilized with Cyclophosphamide 4 g/m^2 and G-CSF 10 microg/kgTT and in all cases conditioning was up to standard BEAM regimen with addition of ATG. EDSS was estimated 3, 6, 9 and 12 months after the procedure. *Results.* With the median follow up of 21 months (10-55), 1 pt (20%) remained stable without new lesions on MRI and other 4 (80%) have showed improvement according to EDSS by at least 0,5 points. Also, there were no transplant related mortality and engraftment was observed in each case of this small group of pts. *Conclusion.* This modest retrospective analysis shows benefit of autologous SCT in pts with secondary progessive multiple sclerosis. Larger number and adequate pts selection with longer follow up is necessary for more valid estimation of efficacy and safety of this therapy option.

1681

IS THERE POSSIBILITY TO IMPROVE TREATMENT OF REFRACTORY CHRONIC EXTENSIVE GAFT VERUS HOST DISEASE

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Introduction. Chronic graft *vs.* host disease (cGvHD) is main determination of long term outcome after transplantation. Similarities in the clinical features with several autoimmune diseases are due to B lymphocyte (B-Ly) disregulation (caused by Th2 cytokines produced by CD4+ donor cells) which is responsible for abnormal production of various auto antibodies. Bad response of cGvHD to different immunosuppressives and fact that chimeric monoclonal anti CD20 antibody (MOAB) induces "*in vivo* depletion" of B-Ly makes this agent interesting as attempt to control refractory cGvHD. *Objective.* Estimating efficacy and safety of MOAB in the therapy of extensive cGvHD refractory to previous treatment approaches. *Methods.* 6 patients (pts) have received MOAB after failure of standard treatment (Methylprednisolone-MPDN, Cyclosporine A- CsA, Mycophenolate mofetil- MMF, Azathioprim- AZA, PUVA[3DOTS]) Pts characteristics were 4 males/ 2 females, median age was 29 years (18-49). Concerning primary diseases 2 of them had ALL, 3 AML and 1 hypoplastic MDS. All of them have received HLA identical sibling transplant with stem cells from peripheral blood in 4 and bone marrow in 2 cases. Despite GvHD prophylaxis with CsA, Methotrexate and MPDN, 4 of them had acute GvHD grade 1-2 and all of them had extensive cGvHD with lichenoid, sclerodermic or pemphyoid skin involvement, oral mucositis and sicca syndrome. Extracutaneous manifestation included lung in 4, liver in 3 and thrombocytopenia in 3 cases. One pt had CMV infection prior to exacerbation of cGvHD. We applied MOAB in recommended regimen (375 mg/m^2 weekly for 4 weeks). *Results.* Exact responses were measured with Schirmer test, spirometry, liver function parameters and hematological findings and response was observed in 5 pts (83,3%) even after first application. It seems that best results were achieved in skin and mucosal improvement (significant reducing of sicca syndrome, improvement of skin turgor and reconstitution of lichenoid and pemphigoid changes). Lung and liver function were improved but it should be mentioned that other immunosuppressants were also given (particularly MMF and AZA). Two pts had severe infectious complication, one of them have died from sepsis after second application, and other developed abcessus pulmonalis in fourth week of treatment. *Conclusion.* Our preliminary data suggests that MOAB in treatment of extensive refractory cGvHD is encouring but needs more investigation at larger number of pts.

1682

SOCIAL BENEFIT IMPROVING ACCESS TO AUTOLOGUS TRANSPLANTATION WITH HEMATOPOIETIC STEM CELL: THE EXPERIENCE OF ONCOLOGY DEPARTMENT OF LECCO (ITALY)

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Background. HSC harvest and storage are not widely available in all hospitals and patients are usually referred to large Hematology Department often located in large cities far from their homes with a significant distress for patients and their families although high dose chemotherapy (HDC) with HSC rescue has definite indications in hematologic malignancies. *Aims.* Considering that the hematologic malignancies range at Lecco's hospital has registered twice as much the number of cases in the last years, it appears more and more important for citizens from Lecco to undergo the HDC with HSC rescue at their local hospital without being obliged to reach hospitals too far from their homes. *Methods.* Oncology Department of Lecco in collaboration with the Haematology Unit of Niguarda Hospital in Milan started a cooperative protocol of HDC-HSC rescue intending to offer these procedures to patients living in Lecco with multiple myeloma (MM) or lymphomas (Hodgkin's, HD, or Non Hodgkin's, NHL) for whom indication for HDS-HCS rescue was agreed, underwent remission induc-

tion. HSC mobilization and peripheral $CD34^+$ monitoring was performed at Oncology Department of Lecco while harvesting and storage at Niguarda Hospital in Milan. HDC with HSC rescue was then performed either at Niguarda Hospital or at Lecco Hospital: in the latter case HSC were transported and reinfused at Lecco Hospital one day following HDC. *Results.* 50 patients (27 male and 23 female), median age 51(range 23-72 years), 23 multiple myeloma (MM), 13 high grade non-Hodgkin lymphoma and 6 non-Hodgkin's lymphoma (NHL), 7 HD in first relapse and 1 LLC entered the protocol mobilization consisted of DCEP for MM, DHAP for NHL and HD. A total of 80 leukapheresis (1-4 per patient, median 1) have been performed and a median 9,3 (4-39,3)$\times 10^6$/Kg of HSC were stored. HDC with HSC rescue has been delivered to 32 patients (8 MM double transplantation, 15 MM single transplantation and 8 NHL and 6 HD single) either at Niguarda Hospital (myeloablative, 27 procedures) or at Lecco Hospital (8 myeloablative and 10 non-myeloablative procedures). *Conclusions.* Based on our experience, HDC with HSC rescue is feasible also in an Oncology department which does not have facilities for HSC harvest and storage provided a strict cooperative protocol with a referral center is agreed. Patients from Lecco area received full HDC with HSC rescue with reduced logistic problems and limited distress for patients and their families and the resulting social benefit is remarkable to with little expenses from the Hospital services maximizing.

1683

REDUCED-INTENSITY CONDITIONING ALLOGENEIC HAEMATOPOIETIC STEM CELL TRANSPLANTATION: AN 8-YEAR EXPERIENCE

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Background. Reduced-intensity conditioning allogeneic haematopoietic stem cell transplantation (RIC-SCT) is a potentially curative option for patients with haematologic neoplasms that can not undertake an allogeneic haematopoietic stem cell transplantation with conventional myeloablative conditioning. *Aims.* To report overall and relapse-free survival at 12 and 24 months after RIC-AlloHSCT, as well as the most prevalent complications of this procedure in a reference center. *Methods.* Retrospective study of consecutive patients treated with RIC-AlloHSCT in our center (Hospital Ramon y Cajal, Madrid), from the years 2000 to 2008. We recorded demographic, clinical and therapeutic variables. *Results.* There were 34 patients, sex ratio 1:1, average age 46,3 (range 18-65) year-old. Number of cases per diagnostics was 8 MDS/AML, 3ALL, 6 Hodgkin's lymphoma, 8 high-grade non-Hodgkin lymphoma, 3 CLL/low-grade non-Hodgkin lymphoma, 4 MM, 1 CML and 1 idiopathic myelofibrosis. Sort of donor was related 94%, unrelated 6%. Previous chemotherapy lines 3 (1-8), previous status (partial remission 41%, complete remission 50%), sort of conditioning Flu/Mel: 68%, Flu/Bul: 26%, Flu/Mel/ATG: 6% and graft-versus-host disease prophylaxis CSA 12%, CSA + MTX 88%. Most common complication was graft-versus-host disease (GVHD) (53%, grade III/IV 27%). Chronic GVHD was 47%. Infections occurred in 56% (12% invasive fungal infection, 21% cytomegalovirus reactivation and 12% both of them). Main mortality causes were infections (53%), GVHD (18%), and disease progression (18%). There was only one conditioning related decease. First year and second year overall survival was 52.5% and 40.4% respectively. Relapse-free survival after 1 year was 72.6%. 60% of deceased patients were in complete remission. *Conclusions.* RIC-SCT presents an overall and relapse-free survival that makes it an option for patients that are not candidate for conventional SCT. In our series, best results were achieved in MM, non-Hodgkin lymphoma and MDS/AML.

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ACUTE LEUKEMIA IN PEDIATRIC PATIENTS: THE CLINIC DATA OF A SINGLE CENTER

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Background. Analyzes the data of 52 consecutive transplants of allogeneic bone marrow, of which 46 donor family and 6 from unrelated donor in patients with acute leukemia. *Aim and methods.* In 50 children, 52 transplant have been performed, 30 for Acute Lymphoblastic Leukemia (ALL) and 22 for Acute Myeloid Leukemia (AML). The diseases for which have been used for transplantation are as follows: for the ALL group 17 relapse, 5 LLA-T, 5 High Risk, 2 infant, 1 resistant; for the AML group 3 M1, 1 M2, 6 M3, 3 M4, 7 M5, 2 M6. For the group ALL 23 patients was male, 5 female; for the AML group were 12 males and 10 females. The mean age was 11.5 years for ALL (8 months-17 years) and 9 years for AML (23 months-15 years). The donors were 24 males and 4 females in the group ALL, while 12 males and 10 females in the AML group. Conditioning protocols were TBI-TY-EDX and TBI-VP16-EDX for the ALL group , BU-EDX -LPAM for the AML group. Immunosuppressive therapy with cyclosporine has been implemented for all patients, in 6 cases (MUD) was also used MTX and ATG. *Results.* The take was achieved, as regards the PMN to 14 days for ALL (range 11-80), 13 days for AML (range 11-28), with regard to the platelets to 22 days for ALL (range 11-112) and 25 days for AML (range 14-35). One patient with ALL has not reached the take. Mucositis was grade I-II in 21 patients (70%), grade III-IV in 5 patients (16,6%) for the ALL group; I-II in 13 patients (59%), grade III-IV in 8 patients (36%) to the AML group. The overall TRM was 11%. In 8 patients there was acute Graft *vs.* Host Disease(GVHD) III-IV grade, 5 patients (16%) for the ALL group, 3 patients (14%) to the AML group; while with regard to chronic GVHD in 6 patients occurred in limited form, in 6 patients extensively. There were no differences in the distribution in the two groups as regards the c-GVHD. The event free survival, with a median follow-up of 7 years, was 60% in the ALL, and 82% in the LMA group. The overall survival is 70%. *Conclusions.* our experience, although on a limited number of patients, confirms that the bone marrow transplant is a valid tool for the treatment of serious disease such as acute leukemia, otherwise fatal. Although burdened by a relapse and a significant GVHD the transplantion for LLA, it should however be taken into account in the patients who do not have other therapeutic possibilities. For the AML the transplant represent the only chance of healing for our patients.

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CXCR7 REGULATE CXCR4/SDF-1 MEDIATED HEMATOPOIETIC SUPPORTING ACTIVITY OF STROMAL CELLS BY CONTROLING EXTRACELLULAR SDF-1 CONCENTRATIONS

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Stromal cell-derived factor 1 (SDF-1)/CXCR4 axis plays a major role in regulating the interactions between hematopoietic stem and progenitor cells (HPSC) and their stromal microenvironment within the bone marrow. A second SDF-1/CXCL12 receptor, CXCR7 binds SDF-1 with high affinity but little is known about its function in hematopoiesis. In the present study, we demonstrate that the activity of CXCR7 is crucial for proper maintenance of hematopoietic activity on stromal layers. Using quantitative reverse transcription-PCR analysis, we demonstrate that CXCR7 is highly expressed in stromal cells whereas it is at the threshold of detection in hematopoietic cells suggesting that it functions primarily in the stromal microenvironment compartment. In favour of this hypothesis CXCR7 stable expression in hematopoietic cells fails to support SDF-1 induced migration and signalling, in coculture CXCR7 expressing cells could inhibit the migration of SDF-1 induced migration of control hematopoietic cells suggesting that they could titrate SDF-1. Furthermore overexpression of CXCR7 in MS-5 stromal cells leads to a reduction of SDF-1 concentration in the supernatants. Supernatants from these cells had substantially lower efficiency in promoting integrin $\alpha 4\beta 1$-mediated adhesion and migration of Mo7e cells to vascular cell adhesion molecule-1 (VCAM-1) and CS-1/fibronectin than their control GFP counterparts. Moreover, human cord blood CD34+ hematopoietic progenitor cells displayed SDF-1-dependent reduced responses in chemotaxis, transendothelial migration, and up-regulation of adhesion to VCAM-1 when supernatants from CXCR7 expressing MS-5 cells were used compared with supernatants from GFP expressing MS-5 cells. Finally, phenotypically primitive murine cells displayed reduced hematopoietic activity when cultured on CXCR7 expressing MS-5 cells. This reduced hematopoietic activity is partly reverted when recombinant SDF-1 was added to CXCR7 overexpressing MS-5 cells. Taken together, our results indicate that CXCR7 controls the bioavailability of SDF-1 and influences BM cell migration, adhesion and hematopoietic activity. Thus CXCR7 behaves as a decoy receptor regulating the SDF-1 level in BM environment.

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ROLE OF IFNα TARGET GENES IN THE IFNα -INDUCED ACTIVATION OF DORMANT HSCS IN VIVO

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Maintenance of the blood system is dependent on dormant haematopoietic stem cells (HSCs) with long-term self-renewal capacity. Upon injury these cells are induced to proliferate in order to quickly re-establish homeostasis. The signalling molecules promoting the exit of HSCs out of the dormant stage remain largely unknown. We have recently uncovered that in response to treatment of mice with interferon-alpha (IFNα), HSCs efficiently exit G0 and enter an active cell cycle. HSCs respond to IFNα treatment by increased phosphorylation of STAT1 and PKB/Akt, expression of IFNα target genes and up-regulation of stem cell antigen-1 (Sca-1). HSCs lacking either the interferon-a/b receptor (IFNAR), STAT1 or Sca-1 are insensitive to IFNα stimulation, demonstrating that STAT1 and Sca-1 mediate IFNα induced HSC proliferation. To confirm the direct effect of IFNα on HSCs and gain more insight into the mechanism of activation we have performed micro-array analysis on sorted HSCs from IFNα treated mice. This analysis revealed the specific induction of a typical set of known IFN target genes in HSCs from IFNα treated mice, strongly supporting our data that IFNα directly activates dormant HSCs. In addition, expression of several cell cycle genes is altered in IFNα stimulated HSCs, including up-regulation of cyclinB2 and CDK7, as well as repression of Pten and Elavl1, known to stabilize p21CIP1 protein. We will present our newest data on the confirmation of the effect on these IFNα target genes and cell cycle related genes. Moreover, we are examining the role of the different IFNα target genes in the effects of IFNα on HSCs. These data will provide us with more insights into the mechanism of IFNα induced activation of dormant HSCs.

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IL-17 AND FGF SIGNALING INVOLVED IN MESENCHYMAL STEM CELL PROLIFERATION

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Background. Mesenchymal stem cells (MSCs) are widespread in adult organisms and are involved in tissue maintenance and repair, as well as in the regulation of hematopoiesis and immune responses. MSCs have enormous potential for cellular therapies, but also can be used in preclinical models for tissue engineering, biocompatibility studies or in basic studies of cell function and tissue development. Therefore, identifying factors inducing MSCs proliferation/expansion and elucidating associated signaling pathways is of great importance for their further exploration within all these settings. *Aims.* We investigated the effects and signaling of IL-17, a Th17 cell cytokine implicated in modulation of immune and hematopoietic responses, and/or FGFb, a growth factor used in standard procedures for stromal cells isolation and expansion, on murine bone marrow MSCs (mBMMSCs) proliferation and elucidated the signaling pathways involved. *Methods.* mBMMSCs were generated from femurs of CBA mice as plastic-adherent cells and quantified by CFU-F assay. mBMMSCs between passages 2 and 5 were used; their "stemness"was confirmed by the capacity to differentiate into chondrogenic, osteogenic and adipogenic lineages; proliferation was analyzed by the MTT assay, while the signaling events were detected by immunoblotting. *Results.* The data demonstrated that stimulation with IL-17 induced dose-dependent increase, while FGFb supplementation had no significant effect, on the CFU-F colony formation. Surprisingly, the combination of IL-17 and FGFb in culture markedly reduced the average number of CFU-F colonies. Western blotting analysis showed that mBMMSCs used expressed cell surface IL-17 receptor. The results of proliferation assay demonstrated dose-dependent augmentation in cell metabolic activity after exposure of cells to either IL-17 or FGFb, being in good agreement with proliferation values previously reported for treatment of MSCs using a single growth factor; however, the proliferative effects were not additive. We next investigated MAPKs signaling pathways and revealed baseline levels of phosphorylated p38 and ERK1/2, but not JNK, activity in mBMMSCs. FGFb treatment led to increased levels of both the phosphorylated p38 and ERK1/2 MAPKs, whereas IL-17 only induced increased phosphorylation of p38 MAPK within first 15 minutes of stimulation. Interestingly, the simultaneous combination of both cytokines did not change the intensity and duration of FGFb-induced ERK1/2, but augmented the intensity and duration of both IL-17- and FGFb-induced p38 phosphorylation levels up to 60 minutes of incubation. *Conclusions.* The results of our study indicated that IL-17 and FGFb utilize distinctive signaling pathways to induce mBMMSCs proliferation. The lack of cooperative activity of these agents on both the growth of CFU-F and the proliferation of BMMSCs can also be explained through the signaling events obtained. Namely, in cases when the signaling pathways induced by different cytokines overlap with each other or merge on a common downstream effector, one can suppose that poor or even lack of synergistic cooperation between factors could be observed. The results highlight the complexity and specificity of the mutual signaling of multiple growth factors involved in BMMSCs proliferation and also reveal necessary data for achieving better control over the MSCs expansion process.

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SIGNIFICANT UP-REGULATION OF CXCR4 IN CD34$^+$ AND CD34$^-$ MESENCHYMAL STROMAL CELLS IN PATIENTS WITH PRIMARY MYELOFIBROSIS

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Background. Primary myelofibrosis (PMF) is the most aggressive stem cell derived Philadelphia chromosome-negative chronic myeloproliferative neoplasia (MPN), with average survival of 5-7 years and no effective therapy. It is characterized by bone marrow (BM) fibrosis, cytopenias, leukoerythroblastosis, teardrop poikilocytosis, extramedullary hematopoiesis, splenomegaly and elevated number of CD34+ cells in peripheral blood (PB). Understanding of biological abnormalities in PMF may lead to the development of new targeted therapies. Discovery of a single point mutation in the tyrosine kinase JAK2 (JAK2V617F) in MPN has revealed an abnormality contributing to the existence to these diseases, and JAK2 inhibitors are being developed clinically. Mobilization of hematopoietic progenitors from BM to spleen and liver in PMF suggests that alternations in the cross talk between hematopoietic and mesenchymal stromal cells (MSC) may also participate in disease biology. *Aim.* To assess the expression of selected group of genes in CD34$^+$ BM cells, as well as BM-derived MSC, from patients with PMF, which may have a role in the biology of this disease. The genes examined included those involved in cell-stromal adhesion (SPARC, CXCR4), metabolism (COX-2, HIF1α), and differentiation and signaling (Pax5 and Socs3). *Materials and methods.* Mononuclear cells (MNC) from BM aspirates from 20 PMF patients and five healthy donors (used as a control) were cultured in αMEM medium containing 20% fetal bovine serum for 2 weeks. Adherent MSC, as well as nonadherent cells, were then collected and sorted using CD34 microbeads. Total RNA was isolated from sorted CD34$^+$ and CD34$^-$ cells and Q-RT-PCR was performed to measure the expression levels of SPARC, COX-2, CXCR4, FOS, Pax5 and HIF1 transcripts, with β-actin as internal control. Results were analyzed using GraphPad Prism (Kruskal-Wallis test and Dunns test). *Results.* (Table 1). There was no significant change in FOS and HIF1α expression between control and patients' MSC. COX-2 was down-regulated in CD34$^+$ patients' nonadherent cells in comparison to CD34- adherent patients' MSC, but in comparison to control cells there was no significant change.

Table 1. Significant differences in gene expressio.

	Sparc	COX-2	CXCR4	Pax5
Kruskal-Wallis	0.0006	0.0034	<0.0001	<0.0001
CTL NA CD34- (n=6) vs CTL A CD34- (n=3)	0.0170 ↑			
CTL NA CD34- (n=5) vs PA CD34+ (n=11)	0.0004 ↑			
PA CD34- (n=11) vs PNA CD34+ (n=18)		0.0004 ↑		
CTL NA CD34+ (n=5) vs PA CD34- (n=9)			0.0026 ↓	
PA CD34+ (n=9) vs PNA CD34- (n=20)			0.0007 ↑	
PA CD34- (n=9) vs PNA CD34+ (n=20)			P<0.0001 ↑	
PA CD34- (n=9) vs PNA CD34- (n=20)			P<0.0001 ↑	
PA CD34- (n=8) vs PNA CD34+ (n=10)				0.0004 ↑

Abbreviations: CTL NA-Control nonadherent, CTL A- Control adherent, PA-Patients adherent, PNA-Patients nonadherent

SPARC was down-regulated in patients' CD34$^+$ adherent MSC in comparison to CD34$^-$ control nonadherent cells. Pax5 showed statistically significant (P<0.0001) up-regulation in patients' CD34$^-$ adherent MSC *vs.* CD34$^+$ nonadherent cells. CXCR4 was significantly (P<0.0001) up-regulated in patients' CD34$^-$ and CD34$^+$ adherent cells *vs.* patients' nonadherent MSC CD34$^-$ and CD34$^+$. *Conclusion.* The expression of

selected genes differs in CD34+ and CD34- adherent MSC and nonadherent cells from patients with PMF as compared to expression in cells from healthy controls. In particular, patients' MSC have significantly higher expression of CXCR4 gene that encodes a CXC chemokine receptor specific for stromal cell-derived factor-1, suggesting its role in the mobilization of hematopoietic progenitors from BM. CXCR4 is also involved in haematopoiesis, angiogenic activity, apoptosis, T-cell differentiation and phagocyte activation. Further investigation of the CXCR4 gene regulation in both adherent MSC and nonadherent mononuclear cells in PMF is warranted.

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A NOTCH REPORTER LINE FOR THE IN VIVO STUDY OF HEMOGENIC ENDOTHELIUM

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Background. The Notch pathway has been shown to play an important role in the formation of definitive haematopoietic stem cells (HSCs) in both mouse and zebrafish embryos. Signalling from the Notch pathway is activated when transmembrane notch receptors present on the surface of one cell are bound by transmembrane ligands, such as Delta and Jagged, on the surface of a neighbouring cell. This triggers endocytosis of the ligand followed by cleavage of the receptor resulting in the release of the Notch Intracellular Domain (NICD). NICD forms a complex with the transcription factor RBPJk/Suppressor of Hairless (CSL) and Mastermind to activate transcription of downstream target genes. *Aims.* We sought to establish an *in vivo* notch reporter line using the zebrafish as a model and to use this line to further define the role of notch in HSC specification. *Methods.* Previous studies have used the CSL DNA binding sequence to drive expression of GFP in a notch reporter system in mammalian cell lines. We utilised this method to establish a stable notch reporter line in the zebrafish. *Results.* We show that our reporter is specifically responsive to notch signalling and can be used to visualise notch activity in a variety of tissues in both embryos and adults. Notch activity is present in both the ventral and dorsal wall of the aorta at timepoints at which we see HSC development from hemogenic endothelium. Using this reporter line, we have also been able to establish more clearly the position of notch signalling in the hierarchy of pathways regulating hematopoietic stem cell formation in the embryo. *Summary.* Using a novel *in vivo* notch reporter line we have further elucidated the role of notch in establishing HSC identity in the embryo.

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COMPARATIVE ANALYSIS OF TELOMERASE ACTIVITIES OF MESENCHYMAL STEM CELLS ISOLATED FROM DIFFERENT HUMAN TISSUES

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Background. Mesenchymal stem cells (MSC) are defined as non-hematopoietic multipotent adult stem cells isolated form bone marrows as well as from other tissue sources. Possessing many application opportunities in tissue regeneration, treatment of autoimmune diseases and gene therapies, MSC should have considerable proliferation capacity before hand. As the telomeric region of chromosome under goes repetitive shortening after each mitotic division, MSC to be used in clinical applications ought to have considerable telomerase activity (TA) to cover the reduction before mitotic crisis.[1] *Aims.* In this study, it was aimed the determination of TA of MSC isolated form different human tissues and their comparative analysis to reveal the relation between the TA and life span of MSC for the selection of prominent cells in experimental treatment. *Methods.* MSC were isolated from different human tissue sources; endometrium (E), adipose tissue (AT), bone marrow (BM), periodontal ligament (PDL), chondrocyte (C), umbilical cordon blood (CB), and amniotic fluid (AF). The TA was determined by Telomeric Repeat Amplification Protocol (TRAP) following the ELISA assay to quantify the activity of the enzyme isolated from MSC. *Results.* Telomerase activities of seven MSC isolated from different tissues were analyzed (Table 1). Depending on their sources, the MSC showed variable TA: in PDL MSC (Case 1), in AF MSC (Case 3) and in E MSC (Case 2). Even in the unsynchronized culture of BM MSC (in Case 2 and 3), it was demonstrated to have TA in contrast to the information in the literature.[2,3] It was remarkable that CB MSC (Case 1 and 2), which showed high proliferation rates, have TA as high as HeLa cells (immortal cell line derived from cervical cancer cells) and considerably high TA in comparison with those of embryonic stem cells in the literature.[2,4] Convincingly, those cells were different in their growth characteristics. *Conclusions.* Generally, the telomerase activity showed deviations within the MSC depending on their isolated source, the donors' age and their clinical history. In the later passages of the same cell line, the variable rate of decrement of TA was observed in relation with the senescence, which limits the life-span of MSC. Therefore, not only the level, but also the rate of change of TA is important in the selection of the MSC for the potential applications.

Table 1. Relative telomerase activity (RTA) of human MSC (%/μg total protein).

Tissue/ Case	Passage No.	RTA	St. Dev.	Tissue/ Case	Passage No.	RTA	St. Dev.
E / C.2	3	0.76	0.03	BM / C.1	3	0	0
E / C.2	10	0.35	0.01	BM / C.2	3	1.2	0.28
E / C.7	3	0.13	0.04	BM / C.3	3	3.29	0.02
AF / C.1	3	0.39	0.18	PDL / C.1	3	9.29	0.06
AF / C.1	10	3.18	0.16	PDL / C.1	10	0.77	0.28
AF / C.3	3	3.51	0.6	C / C.1	4	3.98	
AF / C.3	4	2.87	1.53	CB / C.1	4	107.7	22.8
AT / C.1	3	2.43	0.22	CB / C.2	3	68.6	12.94
AT / C.6	3	11.76	1.28	Fibroblast	1	0.4	0.26
AT / C.7	3	0	0	HeLa	11	89.31	4.68

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BREAST CARCINOMA CELLS MODULATE PHENOTYPE AND FUNCTION OF HUMAN BONE MARROW-DERIVED MESENCHYMAL STROMAL CELLS IN A NON-CONTACT DEPENDENT MANNER

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Introduction. Many solid tumors, including breast cancers, have a predisposition to metastasize to the bone marrow where they might negatively influence hematopoiesis. Mesenchymal stem cells (MSCs) are the progenitors for all stromal cells in the bone marrow and produce cytokines and chemokines to support hematopoietic stem cells (HSCs). In this study, we investigated in-vitro whether tumor cells are able to modulate the bone marrow microenvironment. *Methods.* Human MSCs (derived from the bone marrow of healthy volunteers and the immortalised MSC line SCP-1) were cocultured with MCF-7 breast carcinoma cells or incubated with conditioned medium of these cells. The proliferation potential was detected by BrdU staining or by MTT assay. Flow-cytometric analysis included MSC, HSC as well as tumor markers (CD34, CD44, CD45, CD73, CD90, CD105, CD146, CD166, CXCR4). The osteogenic differentiation potential was considered by ALP measurement after a two week incubation period with differentiation medium. SDF-1 mRNA levels of MSCs were quantified by real-time PCR and the levels of secreted protein were measured using an ELISA kit. *Results.* MSC/MCF-7 cocultures showed a lower MSC proliferation activity in comparison to MSC control cultures. SCP-1 proliferation was decreased to 52.5% by MCF-7 conditioned medium which suggests that the effect on proliferation is cell contact independent. Furthermore, incubation with tumor cell conditioned medium caused a decrease in the positive fraction and mean fluorescence intensity of CD105 and CD146 on MSCs. Downregulation of CD146 correlated with an inhibition of the osteogenic differentiation potential as shown by ALP levels which decreased to about 30% in the presence of MCF-7 conditioned medium. SDF-1 concentrations in coculture supernatants decreased in a time-dependent manner (on average MSCs 717 pg/mL; MSC/MCF-7 145

pg/mL). Incubation with tumor cell conditioned medium caused also a significant decrease of SDF-1 secretion. In accordance with the SDF-1 protein levels the mRNA levels were downregulated in MSCs incubated with MCF-7 conditioned medium. Interestingly, the effects could be reversed after a medium change to normal DMEM. *Conclusion.* The phenotypic and functional changes induced in MSCs during cocultures with breast carcinoma cells or conditioned medium, respectively, suggest a negative impact on the supportive function of MSC for hematopoietic stem cells. The coculture model chosen allows to mimic the competition between tumor cells and HSC for the stromal niche compartment in-vitro.

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BONE MARROW-DERIVED MESENCHYMAL STEM CELLS CO-CULTURED WITH PANCREATIC ISLETS DISPLAY BETA-CELL PLASTICITY

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Background. Numerous studies conducted to date have indicated that specific stem cells derived from adult human tissues can be reprogrammed in order to differentiate into suitable cell types. Reprogramming may be achieved by changing the microenvironment in which the cells are growing to provide signals that may activate metabolic pathways. Successful experiments were carried out *in vivo* by using the microenvironment of the target cell type. When conditions similar to *in vivo* microenvironment are provided, differentiation of stem cells into the desired cell types with full functionality can be obtained *in vitro*. One of the approaches for *in vitro* differentiation is to use cocultures with the microenvironment of the specific cells of the desired cell type, tissue / organ samples (extracts), extracellular matrix compounds or biologically absorbable materials. *Aims.* To study the direct coculturing effect of rat bone-marrow-derived-mesenchymal-stem-cells (rBM-MSCs) on the pancreatic islets (PIs) to obtain functional islet cells. *Methods.* rBM-MSCs were isolated from the rat bone marrow and cultivated under standard conditions. rBM-MSCs at the passage 3 were plated at the density of 1×10^6 cells into T75 flasks and incubated for 2 d until they reached to 50% confluence. The medium was exchanged with the same medium containing 10 mM of BrdU. After 48 h of incubation at 37 °C and 5% CO2, the BrdU-labeled MSCs were trypsinized and evaluated for viability. rBM-MSCs were directly (with cell-islet contact) cocultured with recovered PIs together with the single cell cultures of those cell cultures as control. The effect of direct cocultures of rBM-MSCs with PIs of the normal rats was investigated by using immunophenotypical and functional methods. The change in the amount of insulin secretion was evaluated as indicator for differentiation of rBM-MSCs. *Results.* At the end of the experiment (approximately 37 d of incubation), the original three-dimensional architectures of all islets were impaired and MSCs mixed with islet-derived cells. In rBM-MSCs/islets cocultures we observed positive for insulin and BrdU Islets secreted insulin into the medium but the secretion has increased in the cocultures due to additive effect of insulin secretion from differentiated rBM-MSCs. The functionality tests by ELISA confirmed that insulin secretion of cocultured MSCs with islets (14.57±0.03 ng/mL) was higher than islets (12.83±1.72 ng/mL).

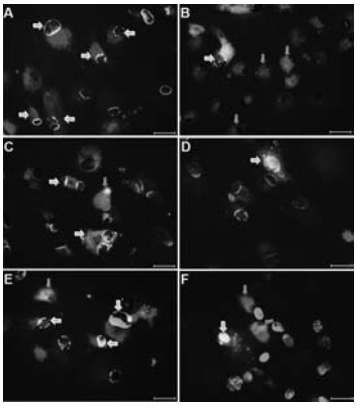

Figure. Coculture of rBM-MSCs with rPIs.

Conclusions. To our knowledge, this is first data that refined these approaches and rather than using pancreatic extracts used well-isolated pure rPIs to achieve differentiation of rBM-MSCs into IPCs. The interaction between MSCs and islets in cocultures initiated differentiation by direct cell-cell contact and by soluble paracrine factors. This approach and similar approaches used by the others will help development of refined well-defined methods for generation of functional insulin-producing cells from stem cells. The development of these methods, however, depends on molecular approaches that will describe novel signal molecules and their associated pathways.

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ROLE OF LEUKOTRIENE B4 IN UPREGULATION OF TRANSENDOTHELIAL MIGRATION OF HEMATOPOIETIC PROGENITOR CELL: MODULATION OF HEMATOPOIETIC PROGENITOR CELL-ENDOTHELIAL CELL ADHESION

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Backgrounds. The mechanisms on hematopoietic progenitor cell (HPC) mobilization by hematopoietic growth factors seem to be multifactorial processes. Though the roles of protease and cell adhesion molecules (CAMs) seem to be important, the precise mechanisms on HPC mobilization are still unknown. Leukotriene B4 (LTB4) is lipid mediator derived from membrane phospholipids during the process of inflammation, and have many roles (ie; inducer of chemotaxis, the production of nitric oxide, transepithelial migration of neutrophil). The major activities of LTB4 include the recruitment and activation of leukocytes, suggesting that it has considerable functional overlap with the chemokine family of chemoattractant peptides, which are known as a HPC mobilizer recently. We previously had reported that LTB4 was able to mobilize HPC of 8.5-fold baseline within only 4 hours without significant side effects in the murine models and LTB4 receptor and reactive oxygen species were involved in the pathway of mobilization induced by LTB4 or G-CSF using C57BL/6 mice. In this study, we investigated the cellular mechanisms of HPC mobilization which was induced by LTB4. *Methods.* We studied the expression of LTB4 receptors (BLT1 and BLT2) on the surface of murine HPC, neutrophil, b.End3 cell (murine microvascular cell line), and MS5 cell (murine stromal cell line) using PCR technique. To elucidate the effects of LTB4 on the cells which compose the bone marrow environment for the HPC mobilization, we studied the influence of LTB4 on proliferation, transmigration, permeability, adhesion and expression of CAMs on HPCs and neutrophils from C57BL/6 mice, MS5 cells and b.End3 cells as well. *Results.* Proliferations and permeability of b.End3 cell and MS-5 cell were not affected by treatment of LTB4. After LTB4 treatment, transendothelial migrations of total nucleated cells (TNC) (22.2% *vs.* 9.4%), HPC (2.7% *vs.* 0%) and neutrophils (75.6% *vs.* 13.6%) from the C57BL/6 mice bone marrow were increased compared to control in transmigration assay. These effects were reversed by LTB4 receptor antagonists, LTB4APA, U75302, and LY255283. Adhesion between HPC and b.End3 cell was increased within 10 minute after LTB4 treatment in adhesion assay. Such phenomena were reversed by LTB4 receptor antagonists. However, The expressions of ICAM-1, VCAM-1, Selectin (E-, L-, P-) on the surface of b.End3 cell, murine microvascular endothelial cell and the expressions of CXCR4, LFA-1 and VLA-4 on the surface of HPC using flow cytometry were not affected by treatment of LTB4. *Conclusions.* Through our data, it is suggested that transendothelial migration of HPCs was upregulated by LTB4 via modulating the adhesion between HPC and endothelial cell. These results suggest that the mobilization of HPC in response of LTB4 may be accomplished by upregulated transendothelial migration of HPC through the modulation of HPC-endothelial cell adhesion in the bone marrow environment. Further studies are necessary to find exact intracellular and molecular mechanisms on the HPC-endothelial cell adhesion by LTB4.

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OSTEOGENESIS IS IMPAIRED IN MESENCHYMAL STEM CELLS OBTAINED FROM FEMORAL HEADS OF OSTEOPOROTIC PATIENTS

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Background. Mesenchymal stem cells (MSC) are the stromal progenitors in bone marrow, and have the ability to differentiate into several

cell types, including osteoblasts, adipocytes and chondrocytes. MSC in osteoporotic patients have scarcely been studied. *Aims.* To isolate and assess the functional capacity and the genomic profile of MSC obtained from femoral heads of patients with osteoporotic fracture compared to the iliac crest counterpart from the same patients. *Methods.* Mononuclear cells (MNC) were obtained after mechanical fragmentations of femoral heads from 20 patients undergoing hip arthoplasty for osteoporotic fracture, together with MNC from iliac crest aspiration from the same patients during the surgical procedure. MSC were isolated following standard *in vitro* expansion in media containing DMEM and 10% fetal calf serum. After third passage, MSC from both sources were tripsinized and used for extensive characterization. This included flow cytometric evaluation of surface markers and *in vitro* multilineage differentiation, with special focus on osteogenesis, that included evaluation of mineralization with alizarin-red staining and PCR of the relevant genes. The presence of genomic changes was studied by array-CGH technology. MSC from osteoporotic fracture and from iliac crest were also compared in terms of microarray expression and confirmed by RT-PCR. *Results.* When compared to MSC from iliac crest, MSC from femoral heads of osteoporotic fractures had a lower expansion capacity. Cells from both sources had similar immunophenotypic pattern and multilineage differentiation ability. Nevertheless, bone mineralization was lower in MSC from osteoporotic femoral heads. In addition, basal expression of markers of osteogenesis such as alkaline phosphatase, osteonectin and type 1 collagen was reduced in this group. No genomic aberrations were detected in MSC from both sources by array-CGH. When gene expression profile of MSC from both sources was analyzed, cells from osteoporotic femoral heads displayed an enhanced expression of negative regulators of osteogenesis (e.g. SFRP1 and 4, PDE1A), and hyperexpression of adipogenesis-inductor genes (e.g. PPAR-gamma, AEBP1). *Summary.* Our study shows that MSC isolated from femoral heads of osteporotic fractures do not harbour genomic aberrations, although these cells have reduced expansion ability and a blockage in their osteogenetic capacity.

1695

SEMI-SOLID DECALCIFICATION AND RESEARCH SYSTEM: A NOVEL METHOD TO STUDY FLUORESCENCE PROTEIN GENE MODIFIED STEM CELLS IN BONE

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Background. It is a great challenge to study the mechanism of stem cells and microenvironment in bone. Gene marking with fluorescent protein (GFP, RFP, etc.) is a powerful technique for stem cells tracing, however, not working well in bone. Why? Bone is such an organ that it is solid, compact, poor lucency and porous. Complex tissues of different characteristics interlace inside bone, such as bone cortex, cells, blood vessels, nerve, matrix and blood, etc. Traditional paraffin embedding and frozen section after decalcification are not proper ways, which may cause the heat injuries in paraffin imbedding, protein denaturation in strong acid decalcification and even the inhibition of antigen-antibody reaction. More important, when bone is decalcified, the cells and other soft tissues inside would move from their original positions without the support of bone frameworks. Glycol methacrylate (GMA) cold embedding technique seems good, but is only suitable for tiny bone. Plastic embedding for hard tissue slicing and frozen section without decalcification maybe better, however, is too expensive to be used widely. *Aim.* To establish a novel, efficient, economical decalcification and research system for the study of stem cells and microenvironment in large bone. *Methods.* Porous and osseous β-tricalcium phosphate (β-TCP) biomaterial scaffold (1×1×0.8 cm) was purchased from Bio-lu Biomaterial Company, China. GFP and RFP gene modified bone marrow mesenchymal stem cells (BMSCs-GFP, BMSCs-RFP) were inoculated in β-TCP scaffolds and cultured for several days. Then, the scaffolds were fixed with 4% paraformaldehyde phosphate and decalcified within an own-made semi-solid decalcification system. After that, 4~8μm frozen sections were made. Histochemical stain, immunohistochemical stain, fluorescence chromosomal *in situ* (FISH), electron microscope, and PCR were tested with those sections. GMA cold embedding technique was used as control. An auto-BMT murine model was also used, bone marrow cells from male FVB-GFP transgenic mice were transplanted into female FVB-RFP transgenic mice, whom was preconditioned with 8 Gy total body irradiation, 4w later, bones of recipient mice were taken out and under investigation. *Results.* During semi-solid decalcification, hard component of the β-TCP scaffold is disappeared slowly, frameworks of the scaffold were replaced gradually by semi-solid substance. After decalcification, position, form, and fluorescence of the BMSCs-GFP and BMSCs-RFP do not change, observed by fluorescence invert microscope, confocal microscopy and frozen sections. Histochemical stain, immunohistochemical stain, FISH, electron microscope and PCR work well in these frozen sections. GMA cold embedding section could also work, but only suitable for tiny bone with a diameter of 2~3 mm. The experiment of auto-BMT GFP to RFP transgenic murine model is still going on. *Conclusion.* This semisolid decalcification and research system make it possible to research thoroughly the location, growth, proliferation, differentiation, migration and interaction of GFP/RFP gene modified stem cells and also the interaction between cells and microenvironment in bone. The on-going study of the GFP to RFP transgenic mice model, especially the changes in the bone, will prove the feasibility of this novel decalcification and research system.

1696

PURIFIED GR1+CD11B+ CELLS INDUCE NEOVASCULARIZATION IN AN ISCHEMIC HIND LIMB MOUSE MODEL

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Introduction and Backgroud. Gr1+CD11b+ cells have been recently described as myeloid-derived suppressor cells that may be involved in tumor angiogenesis. However, there has been little investigation of the role of $Gr1^+CD11b^+$ cells in non-tumor angiogenesis. *Hypothesis.* To study the relationship of $Gr1^+CD11b^+$ cells to neovascularization in an ischemic hind-limb model of C57BL/6 mice, we tested the following hypotheses: (1) The number of tissue-resident $Gr1^+CD11b^+$ cells increases in BM and ischemic muscle after the ischemic injury; (2) prospectively isolated $Gr1^+CD11b^+$ cells can manifest characteristics of endothelial differentiation; and (3) $Gr1^+CD11b^+$ cells modulate neovascularization in ischemic hind-limbs of C57BL/6 mice upon direct injection into ischemic muscle. *Research and observations.* (1) The number of Gr1+CD11b+ cells markedly increased in the ischemic muscle at 4 days post-surgery (n=5 each; $1.08\pm0.65\times10^5$/g tissue in non-surgically treated mice *vs.* $13.43\pm7.52\times10^5$/g tissue in the ischemic muscle, $P<0.05$); then returned to basal level at day 10. (2) Cultured muscle-derived $Gr1dimCD11b^+$ cells aggregated and formed vascular structures in collagen gel culture. In two weeks of Matrigel culture muscle-derived $Gr1dimCD11b^+$ cells expressed endothelial cell makers, CD31 and VE-Cadherin. (3) Following direct injection of purified $Gr1^+CD11b^+$ cells, laser Doppler imaging revealed an increase in blood flow recovery by 14 days post-femoral artery dissection in the cell injected group compared to the control group (n=7 each, 41.14±1.28% in $Gr1^+CD11b^+$ cell injected group *vs.* 35.83±2.57% in control group). *Conclusions.* These results suggest that Gr1dimCD11b+ cells are recruited into ischemic regions after ischemia and may be directly involved in angiogenesis by their capacity to generate the vascular cells.

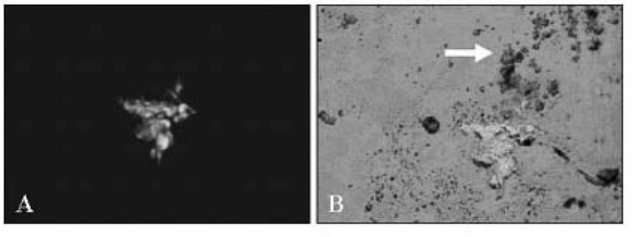

Fig.1 Frozen section after semi-solid decalcification (A. Fluorescence B. Light plus fluorescence)
The original fluorescence, position and form of green fluorescent protein gene modified bone marrow mesenchymal stem cells are preserved in the scaffold after semi-solid decalcification.
White arrow is the residue of bio-material bone scaffold. (×200)

1697

MODELING CHEMOTHERAPEUTIC DAMAGE TO MESENCHYMAL STEM CELLS

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Background. Mesenchymal Stem Cells/Stromal Cells (MSC) form the bone marrow microenvironment and are essential in supporting haematopoiesis. Following stem cell transplantation (SCT), whilst haematopoietic cells are replaced, MSC remain of recipient origin. MSC have previously been shown to be damaged by chemotherapeutic treatment, which is administered prior to SCT. If damage to MSC is severe, it may be implicated in lack of engraftment following some SCT, occurring in 10% of allogeneic peripheral blood transplants, one of the leading causes of mortality in this setting. *Aim.* To elucidate this damage to MSC, a physiologically relevant in-vitro model is needed. This can be challenging as many chemotherapeutic agents are extensively metabolised by hepatic cytochrome P450 enzymes. *Methods.* Several important chemotherapy drugs are administered in an inactive prodrug form, requiring metabolism to the main cytotoxic metabolites (e.g. cyclophosphamide to phosphoramide mustard). The vast majority of agents, however, are administered in an active form e.g. vincristine (VIN) and extensively metabolised by the liver to generally less toxic metabolites. Whereas damaging effects from prodrugs can often be underestimated in-vitro if metabolism is not representative of the in-vivo situation, modelling of damage from drugs such as VIN can easily be overestimated if the agent is not detoxified as in-vivo. Previous in-vitro models of chemotherapy damage have often utilised S9 liver extract, rich in P450 enzymes. However, experiments have raised concerns over the toxicity of S9 itself, particularly over extended periods in culture (e.g. 48 hours), highlighting the need for a more physiologically relevant model. An in-vitro model utilising HepG2 (hepatocyte cell line) spheroids within a co-culture system as a source of metabolic enzymes has been developed. It has been evaluated by analysing MSC morphology, proliferative capacity and expression of adhesion molecules (e.g. CD44) and compared with effects seen in MSC from patients who have received chemotherapy in-vivo (with patient consent and LREC approval). *Results.* The new co-culture model was compared with an S9 model and following 48 hour exposure to cyclophosphamide (CY), expansion of MSC was greatly reduced in the presence of both S9 ($P<0.001$) and HepG2 spheroids ($P<0.01$). However, following 3hr treatment, subsequent expansion of MSC exposed to CY in the presence of S9 was no different to MSC exposed to CY alone, whereas expansion of those exposed in the presence of liver spheroids was significantly reduced ($P<0.05$).In addition, MSC morphology is less uniform and an altered adhesion molecule expression (e.g. CD44) can be demonstrated. When MSC were exposed to VIN alone for 48hrs, morphology was altered, MSC expansion significantly decreased long-term ($P<0.001$) and CD44 expression reduced ($P<0.01$). However, when exposed to VIN in the presence of HepG2 liver spheroids, all of these effects were minimised, with both expansion and CD44 levels less reduced ($P<0.001$ and $P<0.05$ respectively), with CD44 expression not differing significantly from untreated MSC. *Conclusions.* A physiologically relevant model to study chemotherapeutic damage utilising HepG2 spheroids has been developed and results obtained are comparable with effects seen in patients who have undergone chemotherapy treatment for haematological malignancy.

1698

OPTIMIZING THE ISOLATION OF HOMOGENOUS POPULATION OF MESENCHYMAL STEM CELL BY NEGATIVE SELECTION OF CD45, CD11B, CD146 AND TER-119 FROM MURINE BONE MARROW CELLS

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Background. Mesenchymal stem cells (MSCs) can be defined as multipotent cells able to differentiate towards the mesodermal and nonmesodermal lineages. They have shown immunoregulatory and immunossupressive properties by some suggested mechanisms like release of soluble factors (IDO, TGF-β1, IL-10, HGF, PGE2 and HLA-G1) and cell-to-cell contact (PD-L1). Recent reports suggest that MSCs are distributed *in vivo* throughout the body, possibly associated with the blood vessels, interfering in the immunoregulation and peripheral immune tolerance *in vivo*. The plastic-adherent murine MSCs isolated from bone marrow (BM) are heterogeneous populations and murine MSCs cultures remain contaminated by hematopoietic cell populations, like CD11b positive cells, for many passages. *Aim.* Once we are interested in isolating a homogenous MSCs population from NOD mice for gene expression studies of type 1 diabetes by microarrays, the aim was optimizing the isolation of MSCs by magnetic separation of CD45, CD11b, CD146 and Ter-119 from primary culture from NOD mice BM. *Methods.* BM was collected from female NOD mice and cultured at 370C and 5% CO2 until the confluence. Firstly, the primary culture was trypsinized and analyzed by flow cytometry for the expression of CD45, CD11b, CD31, CD117, CD34, CD105, CD90, Sca-1 and CD140b. Then, four plates of primary culture were trypsinized and the harvested cells were incubated with magnetic beads conjugated to anti-CD45, anti-CD11b and anti-CD146 for the immunodepletion. Cell suspension was applied onto the column and the unlabeled cells were collected and analyzed by flow cytometry for the expression of CD45, CD11b, CD31, CD117, CD105, CD90, Sca-1 and CD140b, and the cell viability was analyzed by propidium iodide (PI) incorporation. *Results.* The flow cytometry analysis from primary culture cells showed the percentage of CD45 (85.12±6.17), CD11b (84.38±6.13), CD31 (2.23±1.06), CD117 (13.22±1.89), CD34 (1.96±0.10), CD105 (19.49±10.37), CD90 (9.88±2.59), Sca-1(34.81±0.22), CD140b (10.01±2.20). After the immunodepletion, the percentage of $CD45^+$, $CD11b^+$, $CD31^+$, $CD117^+$ cells was 15%, 13%, 2% and 0.2%, respectively, in the collected cells. These cells showed characteristic adherent fibroblast-like morphology and the expression of mesenchymal markers like CD105, CD90, Sca-1 and CD140b was 45%, 8%, 9% and 19%, respectively. The percentage of collected cells was about 4% in relation to the primary culture. The cell viability of the collected cells was 80%. *Conclusions.* We demonstrated that the isolation of MSCs by magnetic separation from primary culture could reduce the percentage of major contaminant cells and it might be a good strategy for enrichment of a mesenchymal stem cell population for gene expression studies by microarrays. Moreover the viability of isolated cells could make possible to keep a pure culture of mesenchymal stem cells and to make functional or other experimental assays for validation of gene expression studies.

1699

MORPHOTYPES AND PHENOTYPES IN PRIMARY CULTURES BONE MARROW MESENCHYMAL CELL FROM HEALTHY DONORS AND MYELODYSPLASTIC PATIENTS

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Background. Myelodysplastic syndromes (MDS) are clonal disorders affecting hematopoietic progenitor cells (HPC). Despite the relevance of microenvironment in developing MDS, only few studies analyze stromal cell population. *Aims.* We propose an *in vitro* model to study mesenchymal stem cell (MSC) differentiation and functionality and to identify the immunopathogenic mechanisms responsible for the initiation and maintenance of MDS. In order to evaluate MSC niche and the ability of differentiation of these cells, we analyzed 3 normal bone marrows (BM), 5 Refractory Anemias (RA) and 6 Refractory Anemia with excess of blasts (RAEB). We performed: 1. Setting of the appropriate culture conditions for the expansion of MSCs; 2. Morphological evaluation of MSC; 3. Phenotype profile of MSCs antigens and adhesion molecules. Primary MSCs cultures were useful in assessing functional capacity of MSCs compartment because they enable us to identify three different types of cells: multipotent uncommitted MSCs, "committed tripotent-bipotent precursor cells and fibrocytes, mature cells without proliferative capacity. *Results.* Compared to normal BM, in RA we detected a decreased number of committed cells and an increased number of fibrocytes. In RAEB we observed disorganization of architecture, giant amorphous deposits in cultures, a lowest number of uncommitted cells and dysplastic changes of MSCs cells, very similar to those described in the hematopoietic compartment. In addition, there were abnormalities regarding the cellular components of the hematopoietic niche, highlighting the role of other cells, like sinusoidal endothelial and adventitial reticular cells. In conclusion, this data suggests that morphological and phenotypical characterization of MSCs niche is useful in understanding the pathogenesis of MDS.

1700

THE EXAMINATION OF CYTOTOXIC EFFECT OF DENTAL PULP DERIVED MESENCHYMAL STEM CELLS AGAINST A-549 CANCER CELLS

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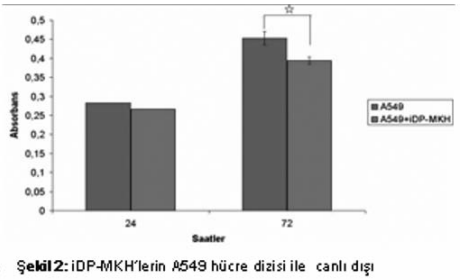
Stem cells are immature, unspecialized cells that are capable of perpetuating themselves as stem cellls and of undergoing differentiation into one or more specialized types of cells .There is strong experimental evience for the notion that cancer develops from cancer stem cells. Cancer stem cells are in many aspects similar to the stem cells. Cancer stem cells have the ability of self-renewal and proliferation, are resistant to drugs, and express typical markers of stem cells. The purpose of cell-based therapy, emerging as a treatment strategy for a good deal of human infections, is to restore, repair or expand the biological role of a damaged tissue or organ. The fundamental biological material used in the studies conducted for this purpose is "stem cells". Due to their immunosuppressive and/or immunomodulator characteristics, mesenchymal stem cells (MSC) today come into use for the purpose of inhibition and/or treatment of many immunodeficiencies in allogeneic transplantations, particularly GVHD (Greft *vs.* Host Disease). Recent reports suggest yet another application for mesenchymal stem cells tecnology: using such cells to immunize against cancer. It was shown that they secrete soluble factor(s) that are capable of inhibiting growth of cancer cells. As a result of the studies toward understanding the mechanism of these immunosuppressive characteristics of MSC's, it was observed that MSC's exhibit potent immunosuppressive activity when they are directly cocultured with Cancer cells, which is believed to arise from cell-cell contact. In our study aiming to research cytotoxic effects of MSC's, human cancer cells were indirectly cocultured *in vitro* with human stem cells isolated as a new cell source for regenerative medicine and immunosuppressive effects of the stem cells on the cancer cells were studied by various methods. For this purpose, MCS's isolated from human embedded dental pulp by enzymatic digestion method were used. For immunophenotypic characterization studies, in flow cytometry device these cells produced positive reaction for CD13, CD44, CD90, CD146 and CD166; reaction was negative for CD3, CD8, CD11b, CD14, CD15, CD19, CD33, CD34, CD45, CD117 and HLA-DR. Cancer-cells (A-549) were maintained in appropriate medium. MSCs in passge 3 were co-cultured with cancer-cells.



Şekil 2: iDP-MKH'lerin A549 hücre dizisi ile canlı dışı koşullarda 24 ve 72 saat süreyle 1:1 oranında indirekt ko-kültür deneyleri sonucunda 72. saatte iDP-MKH'lerin A549 hücreleri üzerinde anti-proliferatif etki gösterdiğini tespit ettik

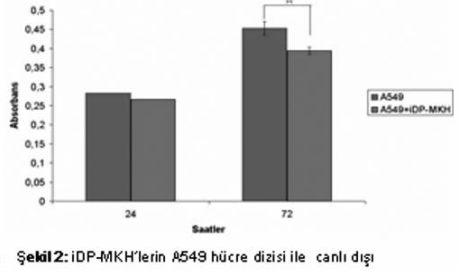


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Figure 1, 2.

MCS's (4×10^5) were transplanted to the bottom of 6-well plates and 2 hour waiting period is left for their adherence. 0.4 micron diameter transwell inserts (main-section) were placed to the wells and 4×105. Cancer -cells were placed in each well (at the ratio 1:1) and taken to in-vitro indirect coculture for a period of 4 days. Following each period of time, activity/proliferation capacity in cancer-cells on the upper side of the intermediate section was measured on spectrophotometer device via MTT method and by evaluating the apoptosis levels after Annexin V-PI staining on flow cytometry device (each experiment was repeated 3 times). After 4-day indirect coculture experiments on hDP-MCS's and cancer cells, we found that hDP-MCS's inhibit proliferation on cancer cells ($P<0.05$) and induce apoptosis ($P<0.05$) (Figure 1, Figure 2). Consequently, initial outputs of our study demonstrated that hDP-MCS's create cytotoxic effects on cancer cells.

1701

BONE MARROW DERIVED MESENCHYMAL STEM CELLS CAN EXPRESS GENES WITH KNOWN ANTI-APOPTOTIC FUNCTION AND CO-STIMULATORY MOLECULES OF ANTIGEN PRESENTING CELLS WITHOUT STIMULATION IN VITRO

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Background. Therapeutic effects of mesenchymal stem cells (MSCs) occur not only by direct differentiation into injured tissue but also by production of paracrine and autocrine factors. MSCs under injured tissue environments can promote the secretion of a variety of cytokines and growth factors that have both paracrine and autocrine activities. Ex-vivo-expanded MSCs isolated from different species, including human, have been shown to suppress the activity of a broad range of immune cells, including T-cells, natural killer cells and B cells. Also, *in vitro* expanded and purified rat MSCs spontaneously secrete transforming growth factor-beta1 (TGF-β1), hepatocyte growth factor (HGF), interleukin (IL)-6, but no interferon (IFN)-γ, IL-4, IL-5 and IL-10. MSCs promote tissue repair by secreting soluble factors that modulate inflammation and angiogenesis. Studies by at least three independent groups have shown that IFN-γ-stimulated MSCs could act as antigen-presenting cells that shown activated effects of MSCs on immune responses. *Aims.* The aim of this study was to investigate the presence of molecules involved in antigen-presentation in addition to genes with known anti-apoptotic functions including mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP2), tumor necrosis factor, alpha induced protein 3 (TNFAIP3) interacting protein 1 and Bcl-3 in rat bone marrow-derived-MSCs (rBM-). We also examined the cytokine and soluble factor secretion by unstimulated rBM-MSCs. *Methods.* rBM-MSCs were isolated from the rat bone marrow and cultivated under standard conditions. Following their characterization, total RNA was isolated from P3 cell cultures and reverse-transcribed into cDNA. The PCR products were analyzed by agarose gel (2%) electrophoresis.

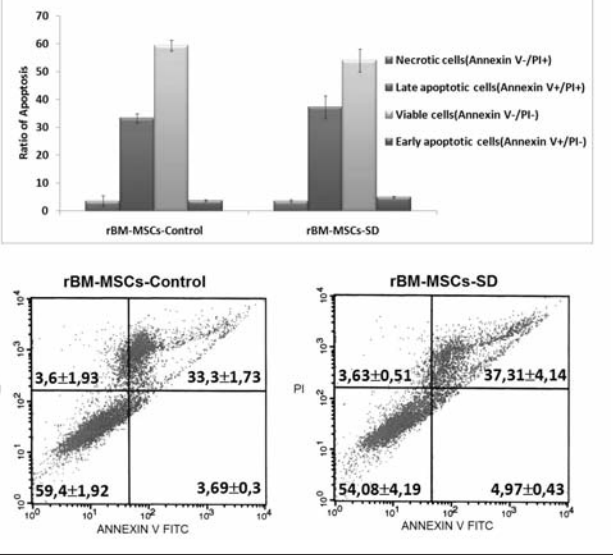

Figure. Serum-deprivation induced apoptosis in rBM-MSCs.

Secretion of rat IL-4, IL-6, IL-10, IFN-γ, TNF-α and TGF-β1 was determined in supernatants by ELISA. rBM-MSCs were seeded at 12×10^3 cells per well in a 96-well plate and then subjected to 24 to 72 hours exposure to serum free medium. Apoptotic cell percentage was detected by flow cytometry with Annexin-V-FITC Apoptosis Detection Kit. *Results.* rBM-SCs did not express CD86 while expressing CD80 which are both counterligands of CD28 with co-stimulatory activity. Interestingly, we determined that besides CD80, rBM-MSCs showed the expression of another co-stimulatory protein CD40. MAPKAP2, TNFAIP3 interacting protein 1 and Bcl-3 were expressed by rBM-MSCs. rBM-MSCs secreted, IL-4, IL-6 and TGF-β1, the secretion was independent from stimulation. But, rBM-MSCs have no IL-10, TNF-α, and IFN-γ secretion. The average percentages of Annexin V+/PI- (early apoptotic) cells were 4.97±0.43 for rBM-MSCs that were incubated serum deprivated medium, and 3.69±0.32 for rBM-MSCs that were incubated complete medium. There was no statistical significance between the two groups (P>0.05). *Conclusions.* Finally, the survivability of rBM-MSCs under serum deprivation may relate with the expression of transcripts with known anti-apoptotic functions. This may highlight the influence of protective role played by anti-apoptotic mechanisms and provide alternative approaches to the treatment of ischemic diseases. The presence of transcripts coding for antigen presenting surface proteins CD 40 and CD80 indicated that these cells were non-professional APCs. In overall, the results of this study emphasized the importance of BM-MSCs in immune system homeostasis along with their regenerative functions.

1702

MAST CELLS AND ANGIOGENESIS IN HAEMATOLOGICAL MALIGNANCIES

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Bone marrow angiogenesis plays an important role in the pathogenesis and progression of haematological malignancies. Growth is halted and a dormancy state is induced in the avascular phase, whereas with clonal expansion and epigenetic modifications of the microenvironment switch to an angiogenic phenotype that generates the vascular phase, and involves changes in the local balance between pro- and antiangiogenic factors secreted by by inflammatory cells and stromal cells. Tumor cells are surrounded by an infiltrate of inflammatory cells, namely lymphocytes, neutrophils, macrophages and mast cells (MCs). Increasing evidence indicates that MCs play a role in tumor growth and tumor-related angiogenesis in both solid and haematological tumors. We have extensively studied the involvement of MCs in angiogenesis in haematological malignancies, i.e. multiple myeloma, B-cell non-Hodgkin's lymphoma, B-cell chronic lymphocytic leukemia and myelodysplastic syndrome. In this context, MCs might act as a new target for the adjuvant treatment of haematological tumors through the selective inhibition of angiogenesis, tissue remodeling and tumor-promoting molecules, permitting the secretion of cytotoxic cytokines and preventing MCs-mediated immune suppression. We have demonstrated that bone marrow angiogenesis decreases significantly in patients with B-cell chronic lymphocytic leukemia who responded to fludarabine therapy, supporting the hypothesis that angiogenesis is a relevant target of therapy in chronic lymphocytic leukemia and providing insight into the role of fludarabine as a potential anti-angiogenic agent. We have further investigated bone marrow angiogenesis in B-cell chronic lymphocytic leukemia patients treated sequentially with fludarabine and low doses of alemtuzumab. Results showed that the complete response rate improved from 45% after fludarabile induction to 90% after alemtuzumab consolidation. Parallely, the extent of bone marrow angiogenesis and the number of MCs decreased continuously after either fludarabine or alemtuzumab.

1703

ROLE OF TUMOR'S CELLS REVEAL IN BONE MARROW IN BREAST CANCER PATIENTS

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Introduction. According to the literature revue, the tumor's cells metastasis into the bone marrow (myelocarcinosis) are diagnosed in 30-90% cases of breast cancer patients. Prognosis for these patients is much worse than for patients without mentioned complication. Thereby, besides the investigation of primary tumor's site, revealing of myelocarcinosis in breast cancer patients could be used for the prognostic estimation of course of decease, as well as in monitoring of systemic therapy *Results.* Therefore, purpose of the investigation was the revelation of atypical cells in bone marrow in breast cancer patients. *Materials and methods.* 48 patients in the age 29 - 66 years with hystomorphologically confirmed diagnosis of breast cancer were considered within our observation. Distribution according to the stage of decease was the following: 0 stage - 1 patient, I stage - 5, IIA - 11, IIB- 12, IIIA - 6, IIIB-7, IV-6. All the patients were carried out a sternal biopsy for the purpose of bone marrow samples investigation and probable revelation of atypical cells of breast cancer metastasis into the site. Pappenheim stain was used for bone marrow samples revue. *Results.* The number of myelocariocytes varied within $45\text{-}187\times10^9$/L. 20 patients (41.7%) had a normocellular bone marrow with a ratio myeloid/erithroid cells 3:1, any abnormal cells were identified in the samples. In 28 (58,3%) patients' bone marrow samples, against the background of hypocellular bone marrow, were revealed a large, round, epithelial cells with hyperbasophilic and vacuolization cytoplasm, interpreted as metastasis cells. According to the stage of breast cancer, patients with myelocarcinosis findings were distributed as follows: IIA - 2 patients, IIB - 2, IIIA - 2, IIIB - 3, IV - 3. *Conclusions.* We confirmed myelocarcinosis in 58.3% of breast cancer patients within our investigation. Therefore, bone marrow biopsy is necessary for all of them to clarify metastasis dissemination and to optimize their future treatment.

1704

PRAME UP-REGULATES MAGE-A3, MAGE-A6, MAGE-A12, A NUMBER OF ANTI-APOPTOTIC GENES AND DOWN-REGULATES PRO-APOPTOTIC AND DIFFERENTIATION-INDUCING ANTI-ONCOGENES

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Background. The preferentially expressed antigen of melanoma (PRAME) is expressed at high levels in large proportion of human malignancies including acute and chronic myeloid, chronic lymphoid leukemia, diffuse large cell lymphoma and multiple myeloma. PRAME is turned out to be an important marker for diagnosis of various malignant diseases and a relevant parameter for monitoring minimal residual disease. In many cases PRAME is a factor of unfavorable prognosis. It was shown recently that PRAME is an inhibitor of RARa protein activity. Nevertheless, distinct elucidation and description of PRAME targets is still necessary in order to reveal a full specter of its tumorigenic properties. *Aim.* Our purpose was to investigate the influence of PRAME on an expression profile of a wide range of genes exploiting a molecular cell model. *Methods.* We cloned PRAME coding sequence under a strong CMV promoter using pCEP4 (Invitrogen) vector. Cell line of human PRAME-negative fibroblasts WI38 was then transfected by PRAME-pCEP4 and pCEP4 itself to obtain both PRAME-expressing variant of WI38 and negative control. Stable PRAME expressing and non-expressing lines of WI38 were selected using hygromycin B. High level of PRAME expression in PRAME transfected line was confirmed by real-time PCR and cell staining with mAb against this protein. Gene expression profiles in these lines were compared by means of microarray analysis using vast panels of dots (25000 genes) and arrays restricted by particular collection of targets (cell cycle and apoptosis regulating genes and transcription factors). Statistical analysis was performed using the dChip software. Selected genes were submitted to real-time PCR confirmation. *Results.* We have revealed that PRAME activation up-regulates MAGE-A3, MAGE-A6, MAGE-A12, membralin (cancer-testis antigens), XIAP, SCC-S2/GG2-1/NDED/ TNFAIP8, BNIP1, RIPK1, acinus, G1P3 (anti-apoptotic genes), SP110 (RARa inhibitor), MAD2L1 (cell cycle regulation), EMP2, IRF9, STAT1 (mitogenic and transcription factors), BOP1 (increases the percentage of multipolar mitotic spindles causing genomic instability) and down-regulates PLAB (genes), PCTK1, TNFAIP2, TNRC6, RNF8 (pro-apoptotic and differentiation anti-oncogenes). *Conclusion.* Our data suggest that PRAME regulates a number of downstream genes in a tumor-enhancing manner. It makes the PRAME to be an important factor of malignisation due to the great diversity of PRAME-dependent genes and their well-known involvement into tumorgenesis.

1705

IN VIVO FUNCTION OF A NOVEL ADAPTOR PROTEIN STAP2 IN THE PROGRESS OF INFLAMMATION

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Background and *Aims.* Signal-transducing adaptor protein-2 (STAP2) is composed of PH and SH2 as well as proline-rich domains, each of which acts as docking sites for several signaling molecules. Our previous *in vitro* experiments have suggested that STAP2 might be involved in multiple steps of inflammatory responses, such as the cytokine production, the mobility of macrophages, and the migration of T-lymphocytes. In the present study, we analyzed *in vivo* effects of STAP2 on inflammation using STAP2-knock out (KO) mice. *Methods.* To confirm the blood component of STAP2-KO mice, we analyzed bone marrow (BM), spleen and thymus samples with flow cytometry. As a model of inflammation, dextran sulfate sodium (DSS)-induced colitis was employed. STAP2-KO and wild-type (WT) C57/BL6 mice aged 15-20 weeks were administered 1.75% or 3.5% DSS (molecular weight 36,000-50,000) dissolved in sterilized drinking water for 7 days. Animals were weighted daily and monitored for rectal bleeding. The severity of colitis was also evaluated by the serum amyloid A (SAA) measurement with ELISA as well as the assessment of colon tissue samples with immunohistochemistry. Briefly, the middle portion of colon tissues were fixed in 4% (w/v) paraformaldehyde, embedded in paraffin, and sliced into 3-mm-thick sections, followed by the staining with hematoxylin and eosin as well as immunohistological staining. *Results.* Flow cytometry analysis of BM cells from WT and STAP2-KO mice showed similar pattern of staining for CD43 and B220 (WT: 4.1% pro-B, 14.5% pre-B, and 8.1% immature B; STAP2-KO: 3.8% pro-B, 17.0% pre-B, and 9.5% immature B). Their BM cells also contained TER119- or Mac1-positive cells similarly. When thymocytes were stained with CD4 and CD8, no difference was observed between WT and STAP2-KO mice (WT: 3.8% DN, 84.2% DP, 9.6% CD4-SP, and 2.5% CD8-SP; STAP2-KO: 4.4% DN, 82.0% DP, 11.2% CD4-SP, and 2.4% CD8-SP). In addition, similar proportion of CD3- or B220-positive cells was detected in their spleen cells. Thus, STAP2-KO mice are likely to have normal blood components, leading us to investigate *in vivo* effects of STAP2 on inflammatory responses. We observed a significant decrease in the body weight of DSS-treated WT mice compared to DSS-treated STAP2-KO mice ($P<0.01$). In addition, DSS-treated WT mice showed rectal bleeding earlier and more frequently than DSS-treated STAP2-KO mice. The shortening of colon length was more obvious in DSS-treated WT mice on day 7. In morphological examinations of colon sections on day 4, the shortening and loss of crypts as well as the infiltration of macrophages (F4/80-positive) and T-lymphocytes (CD3-positive) were demonstrated in WT mice, but not in STAP2-KO mice. The alterations of the colon tissues were observed much earlier and severer in WT mice. Finally, the increase of serum SAA was higher in DSS-treated WT mice than DSS-treated STAP2-KO mice. Thus, DSS-induced colitis becomes less prominent by knock out of STAP2 proteins. *Conclusions.* The development of lympho-hematopoietic cells is independent on the expression of STAP2. However, our experiments using a DSS-induced colitis model have indicated that STAP2 is clearly involved in the progress of inflammation *in vivo*.

1706

MOLECULAR MECHANISMS OF RESVERATROL-INDUCED CELL DEATH IN K562 CHRONIC MYELOID LEUKEMIA CELLS

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Background. Resveratrol is a natural product derived from various plants in order to protect them under stress conditions. Resveratrol has drawn increasingly more attention recently since it affects the processes underlying tumor initiation, promotion and progression. Although we and some other groups have shown antiproliferative and apoptotic effects of resveratrol on chronic myeloid leukemia cells, mechanisms regulated by resveratrol have not been examined in detail. *Aims.* We aimed to investigate the molecular mechanisms of resveratrol induced apoptosis in K562 chronic myeloid leukemia cells. *Methods.* Antiproliferative effects of resveratrol on K562 cells were conducted by XTT cell proliferation assay. Changes in expression patterns of 84 genes involved in apoptosis, cell cycle, senescence, adhesion, invasion, metastasis, angiogenesis, transcription factors, and signal transduction molecules were examined by PCR array in K562 cells exposed to increasing concentrations of resveratrol (10 and 50 μM) for 72 h. *Results.* There were dose-dependent decreases in cell proliferation in response to resveratrol (IC50;80 μM in K562 cells). PCR array results demonstrated that there were more than 3-fold increases in expression levels of 26 and 52 among 84 genes in response to 10 and 50 μM resveratrol, respectively, as compared to untreated controls. (Threshold> 3, $P<0.05$). On the other hand, expression levels of 2 among 84 genes were reduced more than 3 times in response to 50 μM resveratrol. There were overexpression of the genes involved in apoptosis, tumor suppression and cell cycle arrest. The most important ones being TNF, SerpinB5, TNFRSF25, FAS, GZMA, Bax, and Bad. The highest increase (7.5 and 62-fold) was observed in SerpinB5 tumor supressor gene in K562 cells exposed 10 and 50 μM resveratrol comparing to untreated control group. The highest decrease (3.4 fold) was detected in Myc transcription factor genes in response to resveratrol as compared to untreated K562 cells. *Summary/Conclusion.* By this study we explained the molecular mechanisms of resveratrol-induced cell death in chronic myeloid leukemia cells. The results demonstrated that resveratrol triggers apoptosis and inhibits proliferation through regulating cell cycle controller, apoptosis, and tumor suppressor genes in K562 cells. The results of this study strengthen the potential of resveratrol as an anticancer agent for the treatment of cancers including chronic myeloid leukemia.

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1707

UNRESPONSIVENESS OF MDS CELLS TO SDF-1 IS DUE TO PKCZETA INEFFECTIVE CXCR4 RECRUITMENT TO THE PLASMA MEMBRANE

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Background. Myelodysplastic syndrome (MDS) is a clonal hematological disorder that mainly affects the elderly and is characterized by dysplasia in multiple lineages of blood cells, including myeloid, erythroid and megakaryocytic-platelet lineages. MDS is thus believed to result from the malignant transformation of plutipotent hematopoietic stem cells (HSCs). Another important characteristic of MDS is the co-existence of increased cellularity in bone marrow (BM) and cytopenias in peripheral blood. Immature BM cells of individuals with MDS may therefore be defective regarding differentiation or may undergo apoptosis before giving rise to a sufficient number of progeny. Currently the role of the bone marrow niche is being defined as crucial for the correct pluripotency maintenance or differentiation state of HSCs. The chemokine stromal-derived-factor-1 (SDF-1) and its receptor, CXCR4, are thought to develop a main role within the BM microenviroment promoting migration, retention and development of hematopoietic progenitors. Hematopoietic stem and progenitor cells of MDS patients are known to express levels of CXCR4 comparable to normal individuals however their cells do not respond to SDF-1 gradients (Matsuda *et al.,* 2004). Recently, the protein kinase C (PKC) isotype zeta increased activity due to cAMP treatment has been shown to raise CXCR4 expression in $CD34^+$ HPSC (Goichberg *et al.,* 2006). Indeed, PKCzeta act downstream SDF-1 signaling in hematopoietic progenitors through protein G-coupled that activates PI3K, which in turn activates PKCzeta and, consequently, leads towards the activation of proliferation and induction of adhesive or migrating phenotypes (Goichberg *et al.,* 2005). *Aim.* The aim of this study was to identify the poorly express PKCzeta to be the main cause of MDS cells unresponsiveness to SDF-1 gradients, despite CXCR4 being expressed at normal levels. *Methods.* flow cytometry, confocal microscopy, western blotting and transwells migration assays were performed. *Results.* P39 myelomonocytic cell line was used as a MDS model despite having been recently shown to be contaminated with HL-60 cells (Steensma, 2010) and it displayed several MDS-similar behaviors. The unresponsiveness of MDS $CD34^+$ cells to stromal cell derived factor 1 (SDF-1) (Matsuda *et al.,* 2004) was also observed in P39 cells through transwell migration assays using 200 ng/mL SDF-1. We hypothesize that this unresponsiveness to SDF-1 is possibly due to a defective recruitment of CXCR4 to the plasma membrane, as only permeabilized P39 cells exhibited positivity for CXCR4. Furthermore, confocal analysis of P39 cells using a CXCR4 specific antibody showed it to be localized on cell cytoplasm. Western blotting analy-

sis of bone marrow mononuclear cells from MDS patients as well as P39 cell lysates displayed a lack of PKCzeta expression in comparison to $CD34^+$ cells of normal blood donors. *Conclusions.* This work reveals for the first time that the inability of MDS cells to respond to SDF-1 gradients is due to the lack of PKCzeta expression and, consequently, CXCR4 internalization.

1708

PHARMACOGENOMIC VARIANTS OF CYP2C9, CYP4F2 AND VKORC1 AND ITS RELATION TO WARFARIN RESPONSE IN NATIVE OMANI PATIENTS

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Background. The three clinically significant genetic determinants of warfarin inter individual variability are cytochrome P450 [CYP] CYP2C9, CYP4F2 and vitamin K epoxide reductase 1(VKORC1) polymorphisms. CYP2C9 & CYP4F2 polymorphisms alter the pharmacokinetics whereas; the VKORC1 polymorphisms predominantly affect the pharmacodynamic characteristics of warfarin. *Aims.* To study the CYP2C9, CYP4F2 genotypes and VCORC1 haplotypes in the native Omani patients receiving warfarin and correlate them with the dosage. *Methods.* In 214 Omani patients started on warfarin therapy, we assessed six CYP2C9 alleles (CYP2C9*1,*2 ,*3,*4,*5 &*8), two CYP4F2 alleles (CYP4F2*1 & *3) and four VKORC1 nucleotide variants linked to the VKORC1*1, *2,*3 &*4 haplotypes (g.-1639G>A,g.497 G>T,g.698C>T, g.3730G>A,). In patients with warfarin resistance [weekly dose>60 mg] we directly sequenced the PCR amplicons of all the 3 VKORC1 exons, including the intron-exon junctions for the known warfarin resistance associated nucleotide changes.(g.85G>T[p.V29L], g.106G>T[p.D36Y], g.121G>T[p.A41S], g.134T>C[p.V45A], g.172A>G[p.R58G], g.133G>A[p.V66M] and g.3487T>G[p.L128R]) Additionally, demographic and ethnic data, clinical characteristics, response to therapy (as determined by the international normalized ratio(INR)) and steady state warfarin dose requirements were also noted. *Results.* Amongst the patients studied, the mean daily warfarin dose (mg) was 1.0, 3.8, 4, 4.75 and 5.5 in the CYP2C9*3 homozygote, *8 heterozygote, *3 heterozygote,*2 heterozygote and *1 (wild type) respectively (P<0.05). The mean warfarin daily dose (mg) was 2.6, 4.5 and 5.7 in CYP4F2 *3 homozygotes, *1/*3 heterozygotes, and *1 homozygotes (wild type) respectively (P<0.02). When warfarin dose was compared between the VKORC1 A haplotype (*1)[wild type] *vs.* (*3, & *4) the mean daily warfarin dose (mg) was significantly lower at 4.0+1.7 compared to 7.3+1.9(P<0.05) in the former. Similarly, when warfarin dose was compared between the VKORC1 A haplotype (*2) *vs.* (*1) the mean daily warfarin dose (mg) was significantly lower at 2.1+1.7 compared to 4.0+1.7(P<0.05) in the later. In the warfarin resistant group,[weekly warfarin dose > 60 mg; n=8] we identified 2 patients with V66M mutation, one patient with D36Y mutation. In the warfarin sensitive group [weekly warfarin dose<15 mg; n=20] we identified 2 CYP2C9*3/*3 homozygote, 3CYP2C9*3 heterozygote and 11 VKORC1*2/*2 homozygote. We did not find any case with CYP2C9*4,*5, or *2 homozygote in this study. *Summary/Conclusions.* The study confirms that CYP2C9, CYP4F2 and VKORC1 polymorphisms played a significant role in the Omani patients treated with warfarin. Warfarin sensitivity was observed in a significant number of cases and was represented by CYP2C9*3/*3 homozygotes, CYP2C9*3 & *8 heterozygotes, and VKORC1*2/*2 homozygotes. This information is being now used as clinically relevant predictors in this population when practicing pre-prescription CYP2C9, CYP4F2 genotyping and VKORC1 haplotyping to predict at the probable warfarin dose that will be safe and effective for optimal anticoagulation.

1709

MIRNA-29C EXPRESSION IS ASSOCIATED WITH IMATINIB RESISTANCE AND MCL-1 ANTI-APOPTOIC GENE EXPRESSION IN CHRONIC MYELOID LEUKEMIA PATIENTS

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Background. Chronic myeloid leukemia (CML) is a myeloproliferative disease characterized by the presence of the Philadelphia chromosome and Bcr-Abl oncoprotein. Bcr-Abl is a tyrosine kinase (TK) with a deregulated activity which results in leukemic cells proliferation and apoptosis resistance. miRNAs are small non-protein-coding molecules which regulate gene expression at the translational level and have essential roles in cell differentiation, proliferation and apoptosis. Mcl-1 is an anti-apoptotic gene, member of Bcl-2 family, that has been reported as miR-29c target. This miRNA over-expression is also observed in chronic lymphoid leukemia (CLL) and can inhibit tumor formation in an animal model. *Aims.* 1) To verify miR-29c and mcl-1 expression in healthy subjects (controls), CML patients in different phases of the disease, in patients who achieved complete cytogenetic response (CCR) and imatinib (IM) resistant. 2) To correlate mcl-1 mRNA levels with miR-29c expression in CML patients. *Methods.* CML patients' and healthy subjects' peripheral blood mononuclear cells (PBMC) were submitted to RNA extraction by trizol method and reverse transcription was performed with specified primers RT stem loop for each miRNA. TaqMan Universal PCR master mix kit was used for miR-29c expression assay at ABIPRISM 7500 Real Time PCR. SYBRTM Green PCR master mix kit was used for mcl-1 expression assay. The miR-29c and mcl-1 expressions were given as 2-ddCt and URE, respectively. Mann-Whitney and Kruskal-Wallis (Dunns post-test) tests were used for statistical analysis. *Results.* CML patients, in chronic (median=3.24) and advanced phases (median=1.97), presented higher miR-29c expression when compared to control group (median=1.0). However, no difference in miRNA levels was observed among patients in chronic and advanced phases of disease. miR-29c expression was decreased in CML patients resistant to IM (median=1.62) when compared to patients who achieved CCR (median=11.87); (P=0.0010). *Conclusions.* miR-29c might be regulated by Bcr-Abl once its expression was higher in CML patients than in controls. mir-29c expression was associated with IM response. We speculate that miR-29c can modulate mcl-1 expression in IM resistant CML patients.

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1710

DETECTION OF C-KIT D816V IN PATIENTS WITH MASTOCYTOSIS FROM PERIPHERAL BLOOD

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Background. Mastocytosis is a rare disease characterized by pathological mast cell accumulation and activation in tissues such as skin, bone marrow, liver, spleen, and lymph nodes. The clinical spectrum of mastocytosis is variable and may range from a self-limited disease confined to skin in patients to progressive and life-threatening variants associated with poor prognosis. Detection of a codon 816 c-kit mutation is included as a minor diagnostic criterion in the World Health Organization's diagnostic criteria for systemic mastocytosis. Most patients with mastocytosis exhibit the D816V point mutation in the tyrosine kinase domain of the transmembrane receptor protein Kit, leading to its constitutive activation in bone marrow or lesional skin tissue. We studied blood samples from patients with mastocytosis for detection of a codon 816 c-kit mutation. *Methods.* Fifteen patients, nine with systemic mastocytosis and six with cutaneous mastocytosis participated in this study. All patients have serum tryptase level greater than 20 ng/mL. DNA was extracted from peripheral blood and PCR method and RFLPs analysis were applied for the detection of D816V mutation. The amplified region confirmed by sequencing. *Results.* Two patients from the fifteen selected for the study carried the 2447 A>T point variation (D816V) in exon 17 of the KIT gene. One patient was diagnosed for systemic mastocytosis and the other one for cutaneous mastocytosis. *Conclusion.* Detection of the D816V variation in Systemic Mastocystosis patients is important for determining treatment strategy, but as the population of malignant cells carrying this variation is often small relative to the normal cell population, standard molecular detection methods can be unsuccessful. Determining mutational status of the c-kit gene also has pharmacogenomic implications in patients considered for investigational mast cell cytoreductive therapies.

1711

CHARACTERIZATION OF THE C.(-203)A>G VARIANT IN THE GLUCOCEREBROSIDASE GENE AND ITS ASSOTIATION WITH GAUCHER DISEASE PHENOTYPE

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Background. Gaucher disease (GD) is a rare autosomal recessive disorder characterized by defective function of glucocerebrosidase. The main cause of disease is due to mutations of GBA gene. High phenotypic variability has been observed among patients who carry the same genotype, therefore other factors may influence GD phenotype, including polymorphic variants. Previously we have reported the presence of the c.(-203)A>G (g.1256A>G) variant in exon 1 of GBA gene in Spanish GD patients. *Aim.* To determine its role in GD we analyzed the allelic frequency of this change in 206 unrelated Spanish GD patients and a control group (50 samples of cord blood and 100 adult healthy subjects). *Results.* Our results showed the G-allele frequency for GD group was 0.0097±0.0048 and 0.0200±0.0081 for control group, without no statistical significance (P=0.336). In GD patients, G allele was always found in association to another mutation on the same allele and patients carrying c.(-203)A>G variant compared to patients non carriers showed a more severe phenotype. Electrophoretic mobility shift assays demonstrated that the existence of different protein affinity from nuclear extract with mutated promoter with respect to wild promoter. To evaluate whether the variant influenced promoter activity, luciferase reporter plasmids containing 620 bp of the proximal promoter region were constructed. In transient transfection assays, allele G resulted in a 35 % reduction of promoter activity. *Conclusion.* The c.(-203)A>G variant could be a polymorphism and exert a significant effect on GBA promoter activity and might explain in part the phenotype of some GD patients.

1712

CG- AND CNG-TYPES OF MDR1- GENE PROMOTER METHYLATION IN PATIENTS WITH DIFFERENT TYPES OF LEUKEMIA

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The epigenetic changes (DNA-methylation), revealed in the process of leukemia progression affects expression of many genes including multidrug-resistance gene (MDR1). But the exact correlation between MDR1-gene promoter methylation and MDR1 expression remains unclear. The CG- and CNG-comparative methylation status of the 5'-promoter region of this gene in patients with acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML) and myelodisplastic syndrome (MDS) patients was investigated. Totally 39 DNA samples from 28 patients (6 ALL, 14 AML, 4 CML (chronic phase) and 4 MDS (early stage of the disease) obtained from mononuclear cells of peripheral blood and bone marrow were employed in the experiment. We investigated two fragments of MDR1-promoter region with both CG- and CNG- sites presence. Methyl-sensitive PCR method was applied. CG- (HpaII) and CNG- (EcoRII) restriction enzymes were used. Two pairs of primers flanking CG- and CNG-recognition sites of MDR1 gene were constructed for this purpose. 1-st pair: 5'-TAGAGAGGTGCAACGGAAGC-3', 5'-CTCAGGCTTCCT-GTGGCAA-3', 2-nd pair: 5'-AGTCCATGGGGACCAAGTG-3'. 5'-CATCTCCACGAAGGCAGAGTT-3'. For the first time we revealed CNG-type of methylation in cases of hematology malignancies. This phenomena was detected in one ALL, one AML and all MDS and CML cases. In all these cases CG-sites was also methylated. It is interesting to note, that both above-mentioned patients with acute leukemia (ALL and AML) was in complete remission. The rest of DNA samples were CG-hypomethylated. Hypomethylation of MDR1-promoter region is often correlates with overexpression of the MDR1 gene, which is well known as a marker of poor prognosis. This coincides with clinical prognosis estimation of "hypomethylated' patiens. The exact role of both types of methylation of MDR1-promoter region and MDR1-gene expression needs further investigation.

1713

ELEVATED LEUKOTRIENE B4 PRODUCTION BY PERIPHERAL BLOOD MONONUCLEAR CELLS FROM HTLV-1 INFECTED INDIVIDUALS

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Background. Leukotriene B4 is bioactive lipid derived from 5-lipoxygenase (5-LO) pathway of arachidonic acid metabolism, and act by signaling mainly through their high affinity membrane receptor BLT1. It is powerful chemoattractant and pro-inflammatory mediator in several inflammatory conditions. However, the relationship of LT and HTLV-1 infection and related diseases like HAM/TSP is still unknown. *Aim.* The aim of this study was to analyze the production of LTB4 and gene expression profile of enzymes involved in the 5-LO pathway and the high affinity receptor for LTB4 in peripheral blood mononuclear cells (PBMCs) obtained from HTLV-1 infected individuals. *Methods.* PBMCs were obtained from ten healthy donors, eleven subjects with HAM/TSP and ten asymptomatic HTLV-1 carriers (HAC), which were used in cell culture and RNA extraction using Trizol® method. The cells were incubated during 48 hours and LTB4 production was evaluated by EIA after calcium ionophore stimuli. Moreover, cDNAs from PBMCs were synthesized and a quantitative PCR (qPCR) was performed to 5-LO, FLAP and BLT1 genes to determine their relative expression. *Results.* An increased concentration of LTB4 was observed in culture supernatant from asymptomatic and symptomatic groups when compared to health group ($P<0.05$). The level of this lipid mediator was similar between infected groups. Furthermore, PBMCs from HAM/TSP patients constitutively expressed higher levels of 5-LO mRNA than asymptomatic and health donors. In contrast, equivalent amounts of RNA transcripts to FLAP enzyme and BLT1 receptor were observed in cells from both analyzed groups. *Conclusion.* Our data suggest that PBMCs from HTLV-1 infected patients have prominent ability to produce LTB4. These results may be useful for understanding the involvement of arachidonic acid metabolites in pathogenesis of HAM/TSP.

1714

PREVALENCE OF VDR GENE POLYMORPHISM AND ITS ASSOCIATION WITH BONE MINERAL DENSITY IN BETA-THALASSEMIA MAJOR CASES OF NORTH INDIA

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Background. Bone disease in thalassemia is actually a low bone mass, which remains a frequent, debilitating, and poorly understood problem since very long, even in well transfused and chelated pre-pubertal and adult patients. *Objective.* This work was an attempt to delineate calcium status and bone mineral density in a group of transfusion dependent thalassemic adolescents of both sexes, and to correlate it with VDR gene polymorphisms. *Methods.* Bone mineral density (BMD) at both the lumbar spine and hips were measured in 22 adolescents between 10-28 years age of beta thalassemia major by Dual-energy X-ray absorbiometry (DEXA) scanning. The Z score of BMD at lumbar spine and hips were correlated to calcium and phosphorus levels. To find out the prevalence of Vitamin D receptor (VDR) gene polymorphism at exon 2 (Fok1) and exon 9 (Taq1) the Polymerase chain reaction- Restriction Fragment Length polymorphism (PCR-RFLP) studies have been done in 100 thalassemia major cases. Only 22 cases of thalassemia major cases where BMD studies have been performed were estimated for genotype polymorphism of Fok I and Taq I to reveal association. *Result.* Calcium deficiency was found in 31% of Thalassemia patients. However, no significant association of Z score of BMD and Calcium and Phosphorus levels were found. Z-score of BMD at the lumbar spine (-2.3, +/-1.3) was lower than that of the Z-score of the Hips (-2.1, -/+0.89), (P=0.001). Patient with Ff genotype of Fok I site have significantly lower Z- score of BMD at Hips (P=0.034) as compared to patients with FF genotype. However there is no significant change of Z- score of BMD at lumber spine in Fok1 genotype (P=0.46) was observed. We found no

significant change of Z- score of BMD at both Lumber spine and Hips in Taq1 polymorphism. The genotypes frequency for FF, Ff and ff and TT, Tt and tt was 41%, 52% and 7% and 36%, 53% and 11% respectively. On comparing the prevalence of genotypes reported in the literature for north Indians and the prevalence found in present study showed no significant difference. This confirms that frequency and distribution of the polymorphisms in India are though different from other population and ethnic groups but has significant contribution in the field of clinical epidemiological data. *Conclusion.* This is the first report from India, which shows the association of BMD and vitamin D receptor gene polymorphism in thalassemia patients.

1715

TRANSCRIPTIONAL ACTIVITY OF TRANSFORMING GROWTH FACTOR BETA1 (TGF-β1) AND ITS RECEPTORS (TβR-I, TβR-II, TβR-III) AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION

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Background. An increasing use of unrelated and mismatched donors results in a frequent incidence of graft-versus-host disease (GVHD) that continues to be a serious complication following allogeneic haematopoietic stem cell transplantation (alloHSCT). Transforming growth factor beta1 (TGF-β1) seems to be one of the factors that may play a role in the development of GVHD symptoms. *Aims.* The aim of this study was to determine whether transforming growth factor beta1 (TGF-β1), were associated with the occurrence of GVHD in patients who underwent alloHSCT for myeloid leukemias. *Methods.* We investigated the transcriptional activity of TGF-β1 and its three receptors: TβR-I, TβR-II, TβR-III by quantitive real time polymerase chain reaction (Q-PCR) using ABI PRISMTM 7700 (TaqMan). GAPDH gene was used as an endogenous control. *Results.* Fourty adult patients with myeloid leukemias were treated with alloHSCT, 15 from HLA-typical siblings and 25 from unrelated donor. Acute GVHD was observed 20 patients, and 11 patients had chronic GVHD. A prompt decrease in TGF-β1 mRNA expression was demonstrated after conditioning. In patients with acute GVHD, TGF-β1 mRNA expression remained low until day +30 after transplantation. TGF-β1 mRNA expression significantly increased on day +100 in patients who developed chronic GVHD. Multivariate analysis revealed that transcriptional activity of TGF-β1 on day +30 after HSCT is an important factor providing data on the risk of chronic GVHD. No differences were detected in transcriptional activity of TβR-I, TβR-II, TβR-III. *Conclusions.* These results show that low TGF-β1 mRNA expression seem to be associated with the development of acute GVHD and that chronic GVHD symptoms seem to correlate significantly with high TGF-β1 mRNA expression in patients who underwent bone marrow transplantation for myeloid leukemias. Further studies including higher number of patients are needed to establish the role of TGF-β1 in graft *vs.* host disease.

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APPLICATION OF MULTISPECTRAL IMAGING FLOW CYTOMETRY TO CHARACTERIZE NORMAL AND ALTERED HEMATOPOYESIS IN BONE MARROW AND PERIPHERAL BLOOD

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Background. Clinical characterization of normal and altered hematopyesis is mostly performed by separate examination by flow cytometry and light microscopy of bone marrow and peripheral blood samples. The advantages of a system allowing simultaneous determination of immunophenotype and morphology in standard haematological samples are evident. *Aims.* To assess the usefulness of the novel approach of multispectral imaging flow cytometry (MsIFC) in the diagnosis of haematological disorders and rare diseases with haematological profile. *Methods.* Bone marrow and peripheral blood samples from patients undergoing clinical diagnosis at the Haematology Service, for leukaemia, lymphoma or rare disease (mastocytosis, Gaucher's Syndrome) were examined by morphology and processed according to the established multicolor flow cytometry protocols. Aliquots of diagnostic samples plus appropriate single-stained tubes were transferred to the Laboratory of Cytomics for MsIFC. Samples were concentrated by centrifugation and run on an Amnis ImageStream multispectral imaging flow cytometer (Amnis Co., Seattle, USA) using standard acquisition protocols. For each sample 5000 events were stored as .rif files and compensated off line. The corresponding .daf files were analyzed using the interfaced Ideas 4.0 to define fluorescence-based flow cytometric parameters and morpholgy-associated descriptors that could be easily compared to standard phenotype and morphology parameters and also to define new strategies to reveal cell suboppulations of diagnostic relevance. *Results.* Using MsIFC, we have defined in a single-run the appropriate immunophenotypic and morphologyic patterns of normal bone marrow and peripheral blood immune cell subpopulations. In a second step, we have characterized these features on several relevant lymphoproliferative and myelodisplastic syndromes, as well as in some rare diseases, including mastocytosis, hypereosinophylia and Gaucher's syndrome. Also, this approach allowed us to improve the interpretation of ambiguous data in standard immunophenotype and to image rare events. *Summary/Conclusions.* The application of MsIFC to bone marrow and peripheral blood samples prepared as for standard polychromatic flow cytometry is a technically affordable, promising approach to improve diagnosis and translational investigation in oncohaematology.

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DETERMINATION OF THE PATERNAL RHD ZYGOSITY BY TWO DIFFERENT METHODS REVEALED ALTERED RHESUS BOXES

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Background. The RhD gene is flanked by two highly homologous DNA segments of approximately 9000 bp, the upstream and downstream Rhesus boxes. In haplotypes with an RhD deletion, the fusion of the two Rhesus boxes generates the single-hybrid Rhesus box. The discrimination of D+/D+ from D+/D- partners of D- mother with anti D is important to estimate the risk for HDN. This may be achieved by knowing the status of presence or absence of the hybrid rhesus box in the father. *Objectives.* To determine RhD zygosity in RhD positive husband by two different PCR based method. *Methods.* A total of 104 women with RhD negative blood group were recruited for study. Husband's blood group (ABO & RhD), was done in all cases serologically. Blood samples of the husband where blood group was found to be RhD positive were collected in EDTA vial. The samples were tested for the hybrid rhesus box by two different *Methods.* PCR-Restriction fragment length polymorphism (PCR-RFLP) and Sequence specific primer (PCR-SSP). The double blinded methodology was used in the detection. *Results.* Of the 104 women with RhD negative blood group, husband's blood group was found to be Rh D negative in 4 (3.8%). Of the 100 blood samples that were found Rh D positive, 26 were D hemizygous which by both methods were positive for the hybrid rhesus box. No discrepancy was observed in the *Results.* The 4 RhD negative samples were used as control to indicate positive amplification of the RHD deleted allele. *Conclusion.* The precise and rapid detection of RhD zygosity to check the hybrid rhesus boxes is established. As PCR-SSP method included internal control it may be preferable for RhD zygosity determination. Determining paternal Rh zygosity also helps in avoiding unnecessary fetal blood group determination.

1718

IMPLEMENTATION OF AN EFFICIENT WORKFLOW IN CHRONIC MYELOID LEUKEMIA MONITORING IN ROMANIA

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Background. The success of imatinib therapy for chronic myeloid leukemia (CML) has brought new challenges; these include optimizing disease monitoring, imatinib resistance and use of novel, more potent tyrosine kinase inhibitors. Thus, there is a need to establish new best practices for CML management in the post-imatinib era. In Romania imatinib therapy is in progress since 2002, but for some financial reasons the molecular diagnosis is not routinely available so far. In 2007 with research grants support we have set up in our institution a Molecular Biology Laboratory and here we report our results. *Methods.* Total RNA extracted from 2×10^7 cells has been transcribed by RT-PCR into cDNA. For diagnostic samples, the use of Multiplex PCR has been applied to detect simultaneously several kinds of BCR-ABL transcripts. For quantification of BCR-ABL transcript levels a Real-Time Quantitative - Polymerase Chain Reaction (RQ-PCR) using LightCycler® tech-

nology has been applied. Total ABL transcripts were quantified as internal control and results were expressed as the ratio BCR-ABL/ABL. The Nested PCR qualitative method has been used for MRD detection in case of RQ-PCR negative results. *Results.* A total of 535 peripheral blood samples collected from 230 patients with positive BCR-ABL transcript at diagnosis and follow-up has been procesed. Identification of transcript type by Multiplex PCR revealed b3a2 and b2a2 BCR-ABL transcripts in 67% of cases and 33% respectively. In two cases we identified a dual b3a2+b2a2 type. Quantification of BCR-ABL was performed according to the European LeukemiaNet protocol and using a CF=0.7838 for International Scale converssion. From total investigated cases, 133 patients were at first molecular investigation and 97 were in periodical investigation in our laboratory for MRD monitoring. The median of relative quantity of BCR-ABL in the blood before imatinib therapy was 50%. The number of the transcripts in imatinib-sensitive subjects decreased to 2,62% in 6 months. After 12 months of the treatment the BCR-ABL median was 0,038%. Forty patients present a resistance to imatinib with the BCR-ABL range of 12-48% during the treatment. For these patients direct sequencing of the ABL KD was performed. In seven patients we identified 8 mutation types - E450A, Q252H, E450K, E459K, L387M, M244V, F359V and E255K. The association of two mutations per genotype was identified in two patients: L387M + M244V and E450A+Q252H respectively.So this assay generated clinically useful information regarding the therapy with new BCR-ABL inhibitors in our patients. *Conclusions.* It is thought that CML is a rare hematologycal disease, but in Romania it is in increasing frequence. This study, the first in our country, indicates that transcript quantification and mutation screening in BCR-ABL kinase domain could provide practical indications able to direct therapeutic interventions for CML patients. Thus, the molecular monitoring must be an essential part in the clinical management of patients with CML treated with tyrosine kinase inhibitors.

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SCREENING OF AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME (ALPS)

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Background. ALPS is a rare disorder caused by a defective FAS-mediated lymphocyte apoptosis that leads to chronic lymphoproliferation, autoimmune manifestations and increased risk of malignancies. The elevation of double negative T cells (DNTs; $CD3^+$, $CD4^-$, $CD8^-$, TCRα/β+) above the normal upper limit (<1% of lymphocytes) in peripheral blood has been described as a diagnostic criteria for ALPS. *Aim.* to describe the results of DNTs measurement by flow cytometry as a screening test for ALPS in a series of pediatric patients. *Methods.* Samples of patients with unexplained autoimmune cytopenias, organomegaly and/or lymphadenopathy were studied between 2005 and 2009. The measurement of peripheral blood lymphocytes, including CD3, CD4, CD8 and TCRgd subpopulations was assessed by flow cytometry in our laboratory. In those cases with elevated DNTs a blood sample was referred to a specialized laboratory to perform FAS mutation studies by molecular methods. *Results.* A total of 29 samples from pediatric patients (age 1-16 years, 58% males) were studied. The level of DNTs was <1% en 18 cases (62% of the whole series), 1-5% in 9 patients (31%) and 2 cases presented markedly elevated DNTs (8% and 14% DNTs). The latter cases were subsequently found to harbor mutations in FAS gene associated with ALPS. In contrast, no mutations were found in patients with DNTs <5%. The confirmed ALPS cases included a patient who had splenomegaly and hemolytic anemia and the second case presented with splenomegaly and bicytopenia. The first-degree relatives of such cases were subsequently studied, confirming, in both cases, the presence of FAS gene mutations in other family members. *Conclusions.* In our series, FAS mutations were found in two cases with very high level of DNTs (>5%). Screening with DNTs by flow cytometry can be routinely available in most clinical laboratories and is a useful tool when ALPS is suspected.

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THE UTILITY OF EXTENDED WHITE BLOOD CELL DIFFERENTIAL BY FLOW CYTOMETRY IN ROUTINE HEMATOLOGY

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Background. Today's white blood cells (WBC) identification and enumeration by hematology instruments is limited to only six cell types: neutrophils, lymphocytes, monocytes, eosinophils, basophils and NRBCs. Automation of the WBC manual differential is possible by flow cytometry for more accurate and automated information. *Aims.* to evaluate the efficacy of single tube flow cytometry analysis of peripheral blood (PB) for screening different pathological conditions. *Methods.* The CytoDiff™ panel is a 5-color / 6-marker reagent that provides a 9-part cytometric differential from whole blood specimens and comprises CD36-FITC, (CD2+CD294)-PE, CD19-ECD, CD16-PC5, and CD45-PC7 (Beckman Coulter, Inc. (BEC)) and specimens were lysed using VersaLyse (BEC). At least 20,000 $CD45^+$ events per sample were acquired on an FC500 Cytomics flow cytometer (BEC). For each case with elevated numbers of leukocytes or with abnormal cells, additional analyses were performed using light microscopy, full panel extended flow cytometric immunophenotyping and cytogenetics to identify lineages and the level of differentiation of the cells. PB was evaluated from 58 patients with different pathological conditions. Half the patients were females and half males, with adult median age 60. Four boys; median age 10.5, and two girls; median age 4.5 were also evaluated. The study was approved by The Pavlov State Medical University's Institutional Review Board. *Results.* Two cases had no immunological abnormalities in WBC subsets. Extended immunophenotyping determined 13 lymphocytosis cases with more than 50% of nucleated cells (53.1-94.5%) to be B-lymphocytosis, with 12 B-CLL and one PLL. One specimen with normal lymphocytes had high B-cells (45%) and extended immunophenotyping specified it as hairy cell leukemia. Six cases were identified with B-blasts, nine with myeloblasts and two with immature granulocytes. The extended analysis of these specimens identified six B-cell precursors ALL, two cases of MDS transformation to AML, two relapses of AML with maturation, one new diagnosed case of AML with maturation, one case of AML with myelomonoblastic differentiation, three cases of residual disease after AML chemotherapy, one plasmablastic leukemia and one acute promyelocytic leukemia (APL) with t(15;17). Therefore, true positive blasts were identified in 15 of 17 PB specimens using extended immunophenotyping. One case with immature granulocytes in PB was misclassified with the CytoDiff™ panel but verified as plasmablastic and in another case the Imm NE were not normal but abnormal promyelocytes corresponding to an APL t(15;17). The CytoDiff profile in the case of APL was different and may be identified. The final diagnosis was made after bone marrow extended immunophenotyping and cytogenetics. Nine cases had increased numbers of monocytes (14.2-32.0%). Three cases came from the surgery department with more than 20% of monocytes being inflammatory $CD16^+$. Two cases with multiple myeloma after chemotherapy and cytopenia recovery had 38.8% and 43.0% of monocytes being inflammatory $CD16^+$. *Conclusion.* Data demonstrate that a differential leukocyte count using Cytodiff™ allows for verification of a wide spectrum of normal and pathological cells in PB. This will provide clinicians with a diagnostic tool to rapidly determine appropriate therapies for a wide variety of blood-related diseases.

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FLOW CYTOMETRIC ANALYSIS IN PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (H.P.N): A SCREENING OF 161 ALGERIAN PATIENTS

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Background. P.N.H is a consequence of non malignant clonal expansion of one or several hematopoeitic stem cells that have acquired a somatic mutation of PIG-A. The affected cells are deficient in Glycosyl PhospahtidylInositol-anchored proteins (GPI-APs). The variable clinical manifestations are: intravascular hemolytic anemia, venous and cytopenia. The disease often arises in the setting of bone marrow failure syndromes, particularly, aplastic anemia and refractory anemia-MDS. The diagnosis is definitively established by demonstrating the deficiency of

GPI-anchored proteins on blood cells, using monoclonal antibodies. *Patients and methods.* From July 2005 to December 2009, 161 patients were screened for PNH: 110 patients with aplastic anemia (50 new AA, 60 AA under immunosuppressive therapy), 24 patients with myelodysplasia, 12 patients with Coombs negative hemolytic anemia, 10 patients with thrombosis in hepatic veins, 15 Fanconi anemia. We used a 2 color analysis flow cytometry to assess expression of GPI-APs on granulocytes, erythrocytes and monocytes using monoclonal antibodies to CD55, CD59, CD16, CD66b and CD14. *Results.* Evidence of PNH was found in 14 of 110 (37%) patients with aplastic anemia: 29 of 60 (48%) AA under immunosuppressive therapy and 12 of 50 (24%) patients with new AA; 4 of 12 (33%) hemolytic anemia; 5 of 24 (21%) patients with MDS and 4 of 10 (40%) patients with hepatic thrombosis. No abnormal cells were detected in patients with Fanconi anemia. In almost all patients 50 of 54 (93%), the deficiency was partial (PNH II cells). A complete deficiency was found in only 4 patients (2 thrombosis and 2 AA). The PNH clone size ranged from 10 - 96% in AA who undergone immunosuppressive therapy, 7-41% in new AA and on average 38% of PNH granulocytes were found in MDS, 70% in hemolytic anemia and 80% in thrombosis. *Conclusion.* PNH frequently arises in association with disorders of bone marrow failure; particularly in aplastic anemia (37 %in our study). Screening for PNH in patients with AA, even in the absence of clinical evidence of hemolysis is highly recommended at diagnosis since identifying subclinical PNH (PNH-sc) appears clinically relevant. Flow cytometric analysis is the most sensitive and informative assay available for diagnosis of PNH.

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CYP1A1 POLYMORPHISM AND RISK OF LEUKEMIA

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Background. The cytochrome P450 (CYP) enzymes constitute one of the biggest gene families which is involved in the metabolism of both endogenous and exogenous molecules. One of the members of this family, CYP1A1, plays a very important role in metabolizing carsinogens and medications. *Aim.* This case-control study aimed to detect the frequency of CYP1A1*2C polymorphism in Iranian patients with leukemia and racially matched controls. Further, it will help to determine the association of this allele's variants if any, as risk factor to develop leukemia. *Methods.* Our study group consisted of 39 patients with chronic myeloid leukemia (CML), 105 patients with acute myeloid leukemia (AML) and 95 unrelated healthy volunteers as an adult control group. Also 85 children with acute lymphoblastic leukemia (ALL) and 94 age and sex meched healthy individuals as a children control group were involved (informed consent was obtained). Genomic DNA was assayed for restriction fragment length polymorphism in the CYP1A1*2C loci by amplification followed by digestion with BsrDI. The data was analyzed statistically employing chi-square and logistic regression analysis. *Results.* The frequencies of AA genotype (wild type) were 82.05, 62.85, 84.70, 85.10 and 80% in CML, AML, ALL, children and adult control groups, respectively. The frequencies of AG genotype (heterozygote variant) were found to be 17.95, 36.20, 15.30, 14.90, 18.95% in CML, AML, ALL, children and adult control groups. The GG genotype (mutant variant) was 0.95 and 1.05% in AML and adult control groups while it was not observed in CML, ALL and children control groups. Logistic regression analyses showed a significant correlation just between the CYP1A1*2C polymorphism AG and AML patients (OR=2.4 , 95% CI=1.3-4.7, P=0.05). *Conclusion.* A higher frequency of CYP1A1*2C which was observed in AML patients as compared to adult control group, indicates an increased risk for AML in individuals carrying the heterozygote allele CYP1A1*2C. however, the results did not show any association between CYP1A1*2C genotypes and risk of ALL and CML.

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HLA CLASS I AND CLASS II ANTIGEN FREQUENCIES IN DIFFUSE LARGE B-CELL LYMPHOMA

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Introduction. Diffuse large B-cell lymphoma (DLBCL) is the most frequent lymphoma within non-Hodgkin lymphoma (30-50% of all NHL). HLA system class I and II plays an important role in antitumor immune response and lymphoma cells apoptosis. There are some evidences that show a relation between HLA alleles with the incidence and evolution of certain malignant hematological diseases, including B-CLL, pre-B ALL or Multiple Myeloma, between others. Specifically, some works in NHL in certain ethnical groups founds a relationship between the presence of a determined HLA allele with a higher susceptibility to develop the disease. In the same sense, it has been described that the presence and/or absence of certain HLA alleles and/or haplotypes let to identify NHL patients (including DLBCL) with different prognosis. However, up to date there are no studies that analyze these possible associations specifically in DLBCL. *Aim.* To analyze the role of HLA specificities in DLBCL characteristics or behavior. *Patients and methods.* A total of 114 patients diagnosed of DLBCL from a non-biased population from one centre were analyzed. Additionally, a total of 1818 healthy donor individuals from the Castilla y León registry for hematopoietic stem cell-transplantation were included as control population. HLA class I (-A y -B) and II (-DRB1) typing at low-resolution level was carried out according to the standards of the EFI. Allele frequencies were estimated by direct counting. Comparisons of allele and phenotype frequencies between populations were performed with the two-sided Fisher's. P<0.05 were considered statistically significant and it was corrected (Pc) for the number of valid comparisons made (Bonferroni correction). *Results.* Significantly differences among DLBCL patients and healthy control individuals were found. Thus, DRB1*01 phenotypic frequencies were significantly higher in the patients as compared to healthy control individual (34.5% *vs.* 22.3%, P=0.0027, Pc=0.038). In addition, a trend was shown for specificities B*51 (21.9% *vs.* 15.6%, P=0.08, Pc=NS) and DRB1*03 (30.7% *vs.* 23.9%, P=0.09, Pc=NS). *Conclusions.* The present data suggest a possible specific association between HLA and DLBCL incidence. Though, the present study should be considered as preliminary, and higher number of cases is required.

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CELLA-VISION DM96 HEMATOLOGIC ANALYZER EVALUATION IN PATHOLOGICAL SAMPLES

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Objectives. The Cella-VisionTM DM96 (CV) is an automatic system which consists of an automated blood smearing device, a digital microscope and a software. It shows cell images of peripheral blood smears by staining with May Grümwald-Giemsa. Leukocyte differential, erithrocyte morphology and platelet count are performed by the CV. The aim of this study is to evaluate CV functionality in an hematology laboratory, compared to the expert hematologists observation in pathological samples. *Methods.* 50 pathological peripheral blood samples were randomly chosen from Cell-Dyn Sapphire (CD) (Abbott). Of them, 10 showed leukocytosis, 10 monocytosis, 10 lymphocytosis, 10 thrombocytosis and 10 thrombocytopenia. The smear and stain were automatically performed by the Sysmex SP1000. They were also evaluated by CV and by optical microcopic review by an hematologist, afterwards. Differential leukocyte counts given by the CV and CD were compared with the ones given by the cytologist. The estimated platelet count by the CV was compared with the optic and impedance methods given by the Cell-Dyn Sapphire (Abbot). The statistical analysis was peformed by Bland-Altman tests. *Results.* Among the samples which showed lymphocytosis, the CD was the best method in lymphocyte counting, as the CV gave an understimation respect to the expert cytologist view of an 11.4% cells bias. It identified these cells as plasmatic cells, non identified or smudge cells. In monocytosis samples, the best counting method was the CV, as the CD method overestimated the monocyte count respect to expert cytologist with a 5.9% cells bias. These were recognized in most of the cases as blastic cells. In the leukocytosis samples the best counting method for neutrophiles was the CD because the CV method gave an understimation with respect to expert cytologist of a 14.9% cells bias. They were recognized in most of the cases as band neutrophiles. Therefore, neutrophiles count by the CV was overestimated with a 11% cells bias. Related to the thrombocytopenic samples, the optic count and the one given by the CV were the ones with less bias shown. It was higher in the impedance method, which showed an overstimation respect to the optic size (3.9×10^9/mm^3 plt) and in the one given by the CV (5×10^9/mm^3 plt). In the other hand, the thrombocytosic samples counting were overestimated, both impedance and optical methods, with respect to CV. *Conclusions.* In our previous studies, the digital cell autoanalyzer CV gives an appropiate ini-

tial accuracy to the leukocyte formula and platelet count in non-pathological peripheral blood samples. However, according to the results in this study, we believe that an expert cytologist supervision is necessary in pathological samples, specially in those which show atipycal lymphocytosis. The rest of the parameters of this study would not need to be checked by the cytologist.

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THE CHANGES IN HEMATOLOGIC PARAMETERS OF NEWBORNS OF HYPERTENSIVE MOTHERS

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Background. Hypertension is a common disorder in pregnancy. It is the most common reason of maternal and perinatal mortality. Complications are the results of uteroplasental insufficiency. *Aims.* In this study, we investigated the changes in blood parameters including blood cell morphology in newborns of hypertensive pregnant women. *Methods.* Cord bloods of the newborns from 31 hypertensive pregnant women that followed-up at least 3 months and from 32 healthy pregnant women were examined prospectively. Demografic characteristics of mothers and newborns and 1st and 5th minute apgar scores of the newborns are recorded. Complete blood count, peripheral blood smear, reticulocyte count, vitamin B12, folate and ferritin levels and hemoglobin electrophoresis tests were studied. The study was approved by the local ethics committee. *Results.* The age of mothers, the numbers of pregnancy, gestational age, the type of birth, the situations of meconium, nuchal cord, deseleration and neonatal resuscitation, 1st and 5th minute apgar scores, neonatal mortality did not differ between study and control groups. In the study group birth ages are statistically significant lower than the control group (P=0.008). Folic acid, vitamin B12 and ferritin levels did not differ between study and control groups. In the study group, Hb F levels were statistically significant higher (P<0.001) and Hb A levels were statistically significant lower than control group (P<0.001). Both groups showed no significant differences in Hb A2 levels. In the study group, the erythrocyte counts, hemoglobin values, reticulocyte percentages and normoblast counts were statistically significant higher than the control group (P<0.05). There were no statistically significant differences in MCV, MCH and MCHC levels between groups. Leukocyte, neutrophil, lymphocyte, monocyte and eosinophil counts were statistically significant low in the study group (P<0.05). Statistically significant differences were not found in the number of basophils. Although the platelet counts were low, MPV and PDW values were high in hypertensive group. Statistically significant differences were not detected in terms of giant platelet percentages between the two groups. The number of cases with neutropenia and thrombocytopenia in the study group were statistically significant higher than the control group (P<0.001). The two groups did not statistically significant differences in terms of the number of cases with lymphopenia. In both groups, the great percentage of the cytopenic cases was premature. The dysplastic changes in neutrophils and erythrocytes in peripheral blood smears of the study group were more than the control. The number of infections in the study group was statistically significant higher than control group (P<0.05). Increased percentage of the patients with frequent infections initially had neutropenia and also showed disorders of nuclear structure, cytoplasmic granules and cytoplasmic membran abnormalities in leukocytes in peripheral blood smears. Although the blood counts were normal in controls, there were disorders of cytoplasmic granules in peripheral blood smears. *Conclusion.* Neonates of hypertensive mothers should be evaluated for the hematological changes. Complete blood count and peripheral blood smear examination should be used for the early diagnosis of possible hematological complications.

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FLOW CYTOMETRY (FC) IN DIAGNOSIS OF LYMPHOPROLIFERATIVE DISEASES

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Background. A major application of flow cytometry is to help to better characterize lymphoproliferative diseases, providing additional informations to define and specify the monoclonality. *Aims.* Flow cytometry (FC) is one of the most powerful weapon to ensure diagnosis of lymphoproliferative disease; we apply it consistently in our laboratory in complement to cytology and histology. *Materials and methods.* We report the results of 149 patients that have chronic lymphocytosis evaluated by flow cytometry in the hematology department EHS CAC. The methods are based on clinical examination, a cytological study and immunophenotyping performed on a blood sample in 149 patients and a bone marrow sampling in 02 patients; we use a panel of monoclonal AC against lymphoid cells B, T and NK. The results are based on using the Matutes score and the percentage and intensity of positive cells. *Results.* Based on cytological criteria: 03 groups. Group 1: suspicion of CLL in 120 cases: 47 women and 73 men with an average age of 68.7 years (37- 86). The results of flow cytometry: - CLL: 92 patients with a Matutes score: 3 / 5: 03 Pts, 4 / 5: 43 pts and 5 / 5: 46 pts. - Mantle NHL: 10 pts. - Villous NHL: 07 pts. - Follicular NHL: 04cas. - NHL of marginal zone: 02 pts. - Prolymphocytic leukemia: 01. - T leukaemic NHL: 01 pts. - Undefined NHL: 03 pts. Group 2: small cell leukemia NHL: 24 Patients. 11 women, 13 men, average age = 67.18 years (41-81). The results of flow cytometry: - Mantle NHL: 10 pts. - NHL of marginal zone: 04 pts. - Follicular NHL: 04 pts. - Villous NHL: 02 pts. - Undefined NHL: 02 pts. - LLC: 02 pts with a Matutes score: 4 / 5 and 5 / 5. Group 3: suspicion of hairy cell leukemia: 05 pts: 05 men, average age = 50.6 years (36-66). Clinic: spleen: 02 pts, infectious syndrome: 02 pts and an anemic syndrome: 01 pt. Biology: pancytopenia: 02 pts, leucopenia: 01 pt and leucocytosis: 01 case. Bone marrow biopsy: 05 pts with hairy cell leukemia. The results of flow cytometry: Hairy cell leukemia: 04 pts. Normal: 01 pt. *Comments.* In our population, FC specified the nature of lymphomatous monoclonal populations in most patients. Thus immunophenotyping by flow cytometry should be a routine method for diagnosis when lymphoproliferative disease is suspected.

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ASSOCIATION OF WIP1 AND BCR-ABL GENES EXPRESSION IN CHRONIC MYELOID LEUKEMIA

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Background. BCR-ABL fusion oncogene is an activated tyrosin kinase which activates several signaling pathways leading to chronic myeloid leukemia (CML). In addition, the wild-type p53-induced phosphatase 1(Wip1) has been shown to be amplified and overexpressed in multiple human cancer types. *Aim.* This study aimed to determine the relationship between Wip1 and BCR-ABL genes expression in patients with CML. *Methods.* 30 patients in chronic phase of CML were studied (informed consent was obtained). Total RNA was extracted from peripheral blood and cDNA was synthesized accordingly. BCR-ABL gene expression was evaluated by Taqman based Real Time PCR and Wip1 gene expression was assessed by SYBR Green based Real Time PCR. Final data were analyzed statistically. *Results.* In this study, we found a significant correlation between Wip1 and BCR-ABL genes expression in CML patients (P=0.042). *Conclusion.* Our data may suggest a BCR-ABL dependent or independent role for Wip1 in pathogenesis of CML.

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VALIDATION OF RAPID MUTATION SCANNING FOR NUCLEOPHOSMIN (NPM1) MUTATIONS IN ACUTE MYELOID LEUKEMIA

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Background. NPM1 mutations, the most frequent molecular alterations in acute myeloid leukemia (AML) present in up to 40% of patients with normal karyotype, are increasingly important for risk stratification and treatment decisions. Rapid mutation scanning as an alternative to sequencing is of high interest because of the potential for considerable savings in both time and costs. Currently, most reported methods for detecting NPM1 mutations are either technically challenging and/or require additional laboratory equipment. *Aims.* We describe a rapid and simple screening method based on PCR and agarose-gel detection validated on 211 patient samples. *Methods.* Bone marrow or peripheral blood leukemic cells were collected at diagnosis. For RT-PCR analysis, a primer pair was designed that covers exons 7 to 12 of the NPM1 gene. Following FAST PCR, amplicons were visualized on a 2% agarose E-gel, where a double band indicates a mutation and a single band represents the wild-type sequence. All PCR products were verified by direct sequencing. *Results.* Of 211 AML samples analyzed, 101 were NPM1 mutated and 110 were wild-type. Mutations include 93 typical 4-bp insertions (types A, B, D, D7, Gm, G0, J, Nm and IV) and 8 previously

unreported mutations. All 101 positive samples detected by the FAST PCR screening method carried a mutation, and none of the 110 negative cases were mutated, resulting in 100% sensitivity and specificity of the FAST PCR screening method. Dilution experiments resulted in a detection limit of 10% of mutation present in the sample. Of note, a previously undescribed polymorphism in exon 8 was detected in 1 mutated and 5 unmutated samples. *Conclusions.* We conclude that FAST PCR followed by agarose gel electrophoresis is a rapid (less than 2 hours), simple and highly sensitive and specific mutation-scanning technique suitable for the detection of NPM1 mutations. Moreover, this method is inexpensive and can easily be integrated in the routine molecular diagnostic work-up of established risk factors in AML using standard laboratory equipment.

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GLOBAL GENETIC CHARACTERIZATION OF THREE PEDIATRIC AGRESSIVE B-CELL LYMPHOMAS WITH FEATURES OF BURKITT LYMPHOMA

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Background. Burkitt Lymphoma (BL) is an aggressive B-cell lymphoma that is characterized by typical morphological, immunophenotypic and molecular features. The genetic hallmark of this disease is the t(8;14)(q24;q32) and variants resulting in the juxtaposition of the oncogene MYC next to one of the immunoglobulin (IG) loci leading to deregulation of MYC. It is a matter of debate whether MYC-negative BL do exist. Additionally, a microRNAs (mir) expression study of these cases has shown that hsa-mir-34b is down-regulated only in BL cases negative for the MYC translocation. *Aims.* The aim of this study was to perform a complete genetic characterization of three rare pediatric agressive B-cell lymphomas with features of BL but lacking a MYC-break to elucidate their genetic profile and to determine common genetic alterations that could be associated to this phenotype. *Methods.* Three pediatric patients (2 males/1 female, ages: 7, 7 and 16 years) morphologically diagnosed with BL (n=2) or atypical BL (n=1) were studied by conventional cytogenetics (R-banding), Fluorescence *in situ* Hybridization (FISH) and SNP array analyses. One of the patients was also diagnosed with Ataxia Telangiectasia. Hypothesizing a possible hypermethylation pattern of mir hsa-mir-34b in these rare cases, bisulfite pyrosequencing (BPS) assay for hsa-mir-34b was performed in the present series. *Results.* All three cases presented complexly aberrant karyotypes devoid of a t(8;14) or variant. Recurrent cytogenetic changes affected chromosomes 6, 11 and 18. Cryptic MYC translocations were excluded by FISH analyses. SNP array analyses revealed 24, 25 and 17 aberrations per case. In total, 40 chromosomal gains, 6 amplifications and 17 losses were observed. Several commonly altered regions were identified including losses of 6q14.2-q22.2 (2 cases), gains/amplifications of 11q12.2-q23.3 (2 cases), losses of 11q23.3-qter (3 cases) and gains/amplifications of 18q21.1-q22.1 (2 cases). FISH analysis with selected probes confirmed several alterations like gains of ATM and amplifications of BCL2 and MALT. No clear methylation pattern that could explain the down-regulation of mir-34b in these rare cases was observed. Interestingly, in a cytogenetic review performed on pediatric NHL based on cases available on the Mitelman database, from 29 cases diagnosed of BL/Atypical without the t(8;14) translocation or variants, 11 (37%) presented alterations involving chromosome 11. *Summary/Conclusions.* The three MYC-break negative cases diagnosed as BL/atypical BL present common regions of alteration. These regions might harbor pathogenetically relevant target genes involved in the formation of a phenotype resembling Burkitt lymphoma in this rare group of mature aggressive B-cell lymphoma. Moreover, we confirmed by BPS that methylation of hsa-mir-34b is not present in this series, suggesting that other epigenetic mechanism could be involved in the described down-regulation or that the different pattern of expression of mir-34b do not correspond to true MYC-negative BL. Finally, a review the literature describing cases that can be classified as MYC-negative BL/Atypical-BL confirmed that alterations of 11q are recurrent in these cases as observed in the present series.

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QUANTITATIVE ANALYSIS OF MN1 GENE EXPRESSION FOR EVALUATION OF MINIMAL RESIDUAL DISEASE IN CYTOGENETICALLY NORMAL ACUTE MYELOID LEUKEMIA PATIENTS

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Background. Molecular characterization of acute myeloid leukemia (AML) is essential for management of therapy. Molecular markers are necessary for prognostic stratification and monitoring of minimal residual disease (MRD), including early detection of relapse. Unfortunately, only 30% of AML patients (pts) presents a disease-specific molecular signature. So, in the last years, efforts have been made to detect a "universal"marker for AML. WT1 gene is over-expressed in about 80% of all AML cases, and has been successfully used for MRD monitoring. More recently, the meningioma 1 (MN1) gene has been found to be over-expressed in AML with inv(16), and high MN1 levels seems to have prognostic impact in cytogenetically normal (CN) AML pts. *Aims.* To test the possible role of MN1 in AML, we first studied MN1 expression in a group of CN AML pts, evaluating the role of MN1 as marker of MDR in cases over-expressing WT1, by comparing the levels of these two genes at various stage of disease. We then analysed MN1 expression in a group of WT1-negative AML pts, to determine if MN1 could be used for MDR assessment in pts lacking specific molecular markers. *Methods.* To assess MN1 expression, we designed a quantitative PCR assay with TaqMan chemistry, using ABL as housekeeping gene. The assay has been localized on the junction between two exons of MN1. The standard curve was prepared with 5 serial dilutions of RNA from a patient with inv(16) AML; the assay efficiency resulted 0.98. To determine the relative quantification of MN1 expression, we used the 2- Ct method, based on a calibrator obtained on a control group of 12 healthy donors. Then we analysed the expression of MN1 in 23 pts with CN AML over-expressing WT1, comparing the 2 markers at different time points (diagnosis, after chemotherapy, before and after transplantation). Finally, we tested MN1 expression in 11 cases of CN AML with normal WT1 levels. *Results.* Median expression level (2- Ct) of MN1 in healthy controls was 0.37 (range: 0.15-3.44). Among the 23 pts with CN AML and high WT1 expression, the proportion of cases with MN1 level >3.44 was 39% (9 pts), with a median value of 24.72 (range: 3.48-64.74). After chemotherapy, 8/9 pts responded and presented MN1 and WT1 levels within the normal values, while one resistant patient over-expressed both MN1 (17.85) and WT1. One of the eight responding pts relapsed after transplant; both MN1 and WT1 raised, but the former increased to pathological values earlier. Among the 11 AML pts with low WT1 at diagnosis, only one case (9%) over-expressed MN1 (6.76); this patient died during induction therapy. *Conclusions.* MN1 was over-expressed in about 30% of CN AML pts, and its levels correlated well with clinical course and other molecular marker (where available), thus representing a potential candidate gene for MRD assessment. In our small cohort of AML without WT1 over-expression the proportion of cases positive for MN1 was unexpectedly low (only 9%), therefore MN1 could not be used to monitor MRD in pts without other molecular markers.

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MRD LEVELS IN NPM1 MUTATION POSITIVE AML CAN BE ASSESSED WITH EQUAL EFFICIENCY USING EITHER DNA OR RNA OF BONE MARROW MONONUCLEAR CELLS

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Nucleophosmin (NPM1) mutated acute myeloid leukaemia (AML) accounts for 30% of AML. Analysis of NPM1 mutation by real-time quantitative PCR during AML treatment has been shown to be a clinically informative means of analysing minimal residual disease (MRD). Analysis can be performed either from DNA or RNA. Analysis from DNA can be related directly to the number of residual leukemic cells, but this may not be the case for RNA. Expression of mutated NPM1 on diagnostic samples has been shown to be highly variable, as results can vary over 100 fold. It has been unclear whether such high variation remains in the MRD setting and thus how well the remaining mutated NPM1 expression actually reflects the amount of residual leukemic cells. We studied mutated NPM1 levels both from RNA and DNA in diagnosis and follow up samples (n=69) from patients with NPM1

mutation type A (n=24), type B (n=1) or type D (n=2) positive AML. Our aim was to study the correlation of NPM1 levels between the two types of sample material and thus to find out how MRD level measured from NPM1 mutated RNA correlates with the actual load of residual leukemic cells. Bone marrow mononuclear cells were used for total RNA and DNA extractions. Cells were frozen in liquid nitrogen in 107 cells aliquots and stored in -80°C until used for analyses. One microgram of RNA was reverse transcribed to cDNA using random hexamers and M-MLV reverse transcriptase. The NPM1 MutaQuant Kit (Ipsogen, Marseille, France) was used according to the manufacturer's protocol to measure normalised copy numbers (NCN) of mutated NPM1 RNA-transcripts relative to the housekeeping gene ABL transcripts. DNA based quantification of NPM1 mutation was done using in house primers and albumin gene for normalisation. DNA based results were expressed as percentage (%) of MRD relative to patients individual baseline at diagnosis. At diagnosis the median NCN for NPM1 mutated transcripts was 356 but the range was wide (16-930). Most of the follow-up samples that were MRD positive from DNA were MRD positive also from RNA (38/43, 88%). At least two RNA samples were degraded, which may have been the reason for the discrepant results. Quantitative results from the samples positive and measurable by both methods were compared by log-linear regression (R2=0,882) (see figure below). Results show that highly concordant estimates of MRD can be produced from RNA and DNA. The burden of residual malignant cells can thus be estimated even from RNA as source material. The better stability of DNA may, however, favour its use in routine clinical diagnostics.

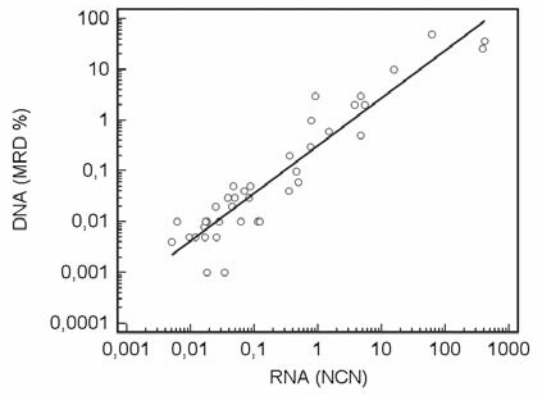

Figure. Correlation of MRD estimates from RNA and DNA.

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IMPROVED DETECTION OF RECURRENT CYTOGENETIC ABERRATIONS USING INTERPHASE FISH ON IMMUNOLOGICALLY IDENTIFIED OR SORTED PLASMA CELLS COMPARED TO CULTURED WHOLE BONE MARROW CELLS

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Background. Multiple myeloma (MM) is a clonal plasma cell (PC) disorder characterized by the early presence of recurrent numerical and structural chromosomal aberrations which can be identified by conventional cytogenetic studies. However, abnormal karyotypes are reported in only 30-40 % of newly diagnosed cases, because of low proliferation rates and limited bone marrow (BM) infiltration. The advent of interphase fluorescence *in situ* hybridization (FISH) allows for an increased detection rate of aberrations, and, in addition, for the identification of recurrent cryptic and prognostically significant changes. Current guidelines based on consensus agreement recommend to perform FISH on PCs. However, FISH is still performed on whole BM in many centres and no comparative data are available so far. *Aim.* The aim of the present study was to assess the impact of different interphase FISH procedures on the detection of chromosomal abnormalities. *Methods.* Two hundred thirty-five BM samples were collected from 225 patients with MM or related PC disorders regardless of treatment status. FISH was performed both on unselected BM cells after a 4 day cytogenetic culture (n=235) and on purified PCs, obtained from fresh BM samples by immunomagnetic selection using anti-CD138 (AutoMACS Miltenyi Biotec,Utrecht, The Netherlands; n=8 or Easy Sep, Stem Cell Technologies, Grenoble, France; n=11) or on BM smears in which PCs were identified by kappa/lambda+ immunostaining (n=216). The low number of cases (n=2), in which both immunomagnetic selection and immunostaining were applied, did not allow a comparison of PC selection techniques. The routine panel included probes for *IGH, RB1, P53* and centromere 9 or BCL6. Other analyzed loci were *CCND1(-XT), MYC, FGFR3, CCND3, TCRαδ/TRAF3* and *PBX1*. *Results.* The following patient and sample characteristics were observed: male/female ratio 122/103, median age 66,8 years (range 35,3-89,4 years) and a median percentage PCs of 32,5 % (range 1-100%). One probe was tested in 129 samples, two probes in 40 samples, three probes in 41 samples and four probes in 25 samples. LSI *IGH*, LSI 13, LSI p53, LSI *BCL6*, LSI *IGH/CCND1*, LSI *IGH/FGFR3*, LSI *MYC* and LSI *IGH/CCNDI-XT* were applied in 197, 85, 79, 41, 16, 4, 3 and 3 samples, and probes for centromere 9, *CCND3, TCRαδ/TRAF3* and PBX1 each in one sample. Of a total of 432 tests, 111 were excluded for further analysis as less than 20 plasma cells could be evaluated per probe. In the remaining 321 tests, FISH on PCs detected abnormalities in 71 % of cases, compared with 35 % of abnormal cases in FISH on cultured whole BM (P<0,0001). Moreover, FISH on PCs allowed to detect more abnormalities per case and identified higher percentages abnormal nuclei (P<0,0001). *Conclusion.* In summary, FISH has a higher detection rate of chromosomal abnormalities if plasma cells are selectively analyzed. In addition, more abnormalities per case could be detected. This study confirms FISH on PCs to be the preferred technique for routine cytogenetic investigation of MM.

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COMPARATIVE STUDY OF DETECTION LIMITS IN 3 DIFFERENT ASSAYS FOR MOLECULAR MONITORING IN CML: STANDARD RQ-PCR, REPLICATE RQ-PCR AND DIGITAL PCR

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Background. Prolonged imatinib therapy leads to a progressive reduction in BCR-ABL transcript levels measured by RQ-PCR assay, and some patients achieve complete molecular response (CMR), which is defined as sustained undetectable BCR-ABL using RQ-PCR assay with a sensitivity of at least 4.5-log below the standardized baseline. As more potent novel tyrosine kinase inhibitors have become available for newly diagnosed chronic phase chronic myeloid leukemia (CML) patients, the number of patients with CMR may increase. However, due to the sensitivity limit of the current RQ-PCR technology, CMR should not be considered as cure as more than 10^6 leukemic cells can still remain in the absence of detectable BCR-ABL transcripts by RQ-PCR, and currently there is no methodology to further classify the patients in CMR. *Aims.* In this study, sensitivity limits of replicate RQ-PCR (rRQ-PCR) and digital PCR (dPCR) assays for measurement of BCR-ABL transcript level was compared to that of standard RQ-PCR assay to assess if more sensitive detection methodology can be implemented for molecular monitoring in CML. *Methods.* Cell line and patient sample dilutions ranging from 10^{-3} to 10^{-7} were used to determine sensitivity limits in standard RQ-PCR, rRQ-PCR and dPCR assays. For preparation of serial dilutions, 10-fold serial dilutions were performed by mixing RNA from K562 cell line (BCR-ABL positive) and RNA from healthy donor peripheral blood mononuclear cells (PBMC) (BCR-ABL negative), and by mixing RNA from one CML patient sample collected at the time of diagnosis (BCR-ABL positive) and RNA from healthy donor PBMC (BCR-ABL negative). *Results.* While standard RQ-PCR assay could detect down to 10^{-5} of cell line dilutions and down to 10^{-4} of patient sample dilutions, rRQ-PCR assay showed 2-log improvement in the detection sensitivity limit by detecting down to 10^{-7} and 10^{-6} of cell line dilutions and patient sample dilutions, respectively. (Table 1) In dPCR assay, no targets were detected from 10^{-6} of cell line dilutions and 10^{-5} of patient sample dilutions, and this sensitivity limit is equivalent to the sensitivity limit of standard RQ-PCR assay. Overall, in terms of detection sensitivity, both standard RQ-PCR assay and dPCR assay showed similar sensitivity limits, whereas rRQ-PCR assay improved the sensitivity by 2 logs. *Conclusions.* In this study, the concept of rRQ-PCR assay was to increase the number of replicates of each sample in RQ-PCR assay, and a 2-log improvement in the detection sensitivity implies that residual disease down to 10^4 leukemic cells can be detected. Patients in CMR who is currently categorized based on the data derived from standard RQ-PCR assay could be classified further using more sensitive methodologies

such as rRQ-PCR assay. Although significant enhancement of sensitivity was not induced in dPCR assay, some optimization procedures might be able to increase the sensitivity, and it would be an attractive alternative to labor-intensive rRQ-PCR assay. These data show the potential of highly sensitive PCR approaches for molecular monitoring, and these approaches examined here can be extended to expand our understanding of molecular profiles in CML patients and to correlate to clinical significance.

Table 1. Comparison of detection limits.

Assays	K562 cell line dilutions	CML patient sample dilutions
Standard RQ-PCR	10^{-5}	10^{-4}
Replicate RQ-PCR	10^{-7}	10^{-6}
Digital PCR	10^{-5}	10^{-4}

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THE TRANSLOCATION T(8;21) IN AML IS TRULY BALANCED AS SHOWN BY ARRAY CGH AT 2.5KB RESOLUTION

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We have previously shown that the t(9;22) in CML is associated with genomic deletions resulting in loss of chromosome 9 and 22 sequences surrounding the translocation breakpoint on the derivative 9 or on additional partner chromosomes or on both. Such deletions were found in 15-20% of the patients studied and the presence of the deletion was associated with poor survival. Previous work has shown the presence of sub-microscopic deletions also in AML patients with t(8;21). These deletions were found in 6% of the patients with t(8;21), affected the der(8) chromosome and span up to 1.3 Mb in size. However 2 of the 6 patients had deletions of 5' sequences of the RUNX1T1 gene but did not carry the classical t(8;21) translocation detectable by FISH with commercial ETO/AML1 probes (Vysis), instead a RUNX1-RUNX1T1 fusion product was identified by RT-PCR. In an attempt to further characterise deletions associated with the t(8;21) we analysed 32 samples that had previously found cytogenetically to harbour a t(8;21) translocation. We employed array comparative genome hybridisation using a customised array. We enriched the areas 10 Mb upstream and downstream of the genes RUNX1 and RUNX1T1 on chromosomes 21q11.2 and 8q22.1 regions respectively achieving a probe density of 2.5 Kb. Further more, we covered the 9q21-9q34 segment with a higher density probes set to verify loss identified by conventional karyotyping. The rest of the probes (60K in total) were distributed evenly across the genome. We were unable to detect any recurrent genome loss within the areas flanking the RUNX1 and RUNX1T1 genes thus proving that the t(8;21)(q11;q22) is truly balanced. Instead, there were a number of unique genome deletions that spanned on average ~250bp within the 8q22 and 21q11 areas, the nature of which remains to be investigated.

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DETECTION OF AN ABERRANT FORM OF E13A2 BCR-ABL TRANSCRIPT CAUSING FAILURE OF MOLECULAR DIAGNOSTICS IN PATIENT WITH CHRONIC MYELOID LEUKEMIA (CML)

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Background. Molecular tools based on RT-PCR became a diagnostics standard for detection of BCR-ABL mRNA transcript in routine practice. A majority of CML patients harbor e13a2, e14a2 fusion transcripts, whereas e1a2 and e19a2 are significantly less frequent. Moreover, also atypical transcripts have been reported. These include breakpoints outside the cluster regions, interposed intronic sequences or genomic breakpoints within individual exons. Unfortunately, these represent an increased risk of miss-interpretation during investigation at the time of diagnosis. *Aims.* Description of an aberrant e13a2 BCR-ABL transcript type detected in patient with CML diagnosed in chronic phase and its role in the failure of standard RT-PCR *Methods.* Design and verification of pacient-specific TaqMan assay for quantitative monitoring of minimal residual disease (MRD). *Methods.* An optimized multiplex RT-PCR for the initial detection of BCR-ABL transcripts was used. In order to verify and validate the ambiguous results from multiplex RT-PCR, a more sensitive two-step nested RT-PCR was performed. The nested-PCR amplicon with unexpected size was subsequently analyzed by direct sequencing. The resulting sequence was compared with sequences of ABL and BCR gene. The segment of unknown sequence was analyzed using BLAST software. *Results.* The standard multiplex RT-PCR failed to identify a BCR-ABL transcript: (a) peripheral blood (PB) was detected as negative, (b) bone marrow (BM) revealed no amplification at all. Two-step nested RT-PCR revealed the atypical PCR products from both materials: (a) amplicon of unexpected size detected in external round and (b) no amplification detected in internal round. Subsequent sequence analysis revealed a joining of BCR gene exon 13 and ABL gene exon 2, with the 17 bp insertion of unknown origin between them. The inserted 17 bp fragment was subsequently identified as 100% homologous to the complementary sequence of ABL intron 1b (GenBank NG012034, bases 25707-25723). Moreover, the sequence analysis revealed the breakpoint within exon 13 of BCR gene at position 3247 (GenBank Y00661), where 56 bp were deleted. As a result, an aberrant BCR-ABL transcript e13a2 with insertion of a complementary sequence of ABL intron 1b, together with deletion within BCR exon 13, was detected (Figure 1). Considering the localization of different breakpoint within the exon 13 of BCR gene, for long-term follow-up, it was necessary to design patient-specific primers and TaqMan probe using Primer ExpressTM v.3 software. *Summary/Conclusions.* In this report we show how easy the standard and well established method for BCR-ABL fusion detection may fail in CML patient. Using the series of additional analyses, an atypical BCR-ABL transcript has been identified and the patient-specific RQ-RT-PCR system for MRD monitoring was subsequently designed. Additionally, we highlight an importance of the detailed investigation in case that unexpected results occur, since even highly standardized and properly used methods can occasionally fail.

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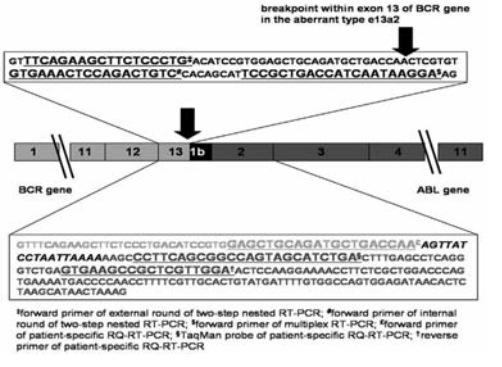

Figure 1. Scheme of an aberrant BCR-ABL e13a2 transcript.

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CHROMOSOMAL ABERRATIONS IN CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS DETECTED BY FISH OR CONVENTIONAL CYTOGENETIC WITH CPG OLIGONUCLEOTIDE STIMULATION - STRENGTHS AND DEFICIENCIES OF TWO METHODS

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Background. Detection of chromosomal abnormalities in chronic lymphocytic leukemia (CLL) patients by conventional cytogenetic (CC) method does not give satisfactory results, mainly due to the low mitotic effectiveness of classical B-cells mitogens. Fluorescence *in situ* hybridization (FISH) is a more sensitive method and allows the detection of chromosomal aberration of prognostic value. However, it is expensive and suitable only for analysis of chromosomal regions with known specific DNA probes. *Aims.* The aim of our study was to compare the effectiveness of CC method with CpG- oligonucleotide DSP30 stimulation (CC-DSP30) with that of FISH in detection of chromosomal aberrations of prognostic value and to answer the question if this method allows the detection of other unknown chromosomal abnormalities. *Methods.* CC-DSP30 and FISH methods were tested in a group

of 62 previously untreated CLL patients (17 women and 45 men; mean age 67 years), after their written consent. Peripheral blood cells were cultured *in vitro* for 72 hours in the presence of CpG-oligonucleotide DSP30 (2μM/mL) (TIBMolBiol, Berlin, Germany). Chromosomes were identified by GTG banding according to standard protocols and classified according to ISCN 2009 nomenclature. In all patients peripheral blood smears were performed for interphase FISH using following locus-specific probes: LSI D13S319 (13q14.3)/LSI 13q34/CEP 12 Probe and LSI p53 (17p13.1)/LSI ATM (11q22.3) Probe (VYSIS, Germany). Metaphase FISH was performed for confirmation of some additional aberrations. To evaluate the agreement between these two methods in detection of the same chromosomal aberration, Fisher's exact test was performed. A P<0.05 was the level of confidence. The measure of agreement was estimated according to the strength of agreement using kappa coefficient. *Results.* Peripheral blood samples were successfully karyotyped in 56/62 (90%) patients. Abnormal karyotype was established in 41/56 (73%) patients by CC-DSP30 and in 52/62 (84%) by FISH. In 15/41 (37%) cases karyotype was complex (3 or more abnormalities). Deletion 13q14 was detected in 10/56 (18%) patients by CC-DSP30 and in 41/62 (66%) by FISH (P=0.01, P=0.18). In the karyotype of 12/26 (46%) patients with sole del(13)(q14) detected by FISH, the CC-DSP30 showed other numerical and structural aberrations. In this group of patients 4/12 (33%) exhibited complex karyotype. CC-DSP30 allowed to detect deletion 11q22 in 14/56 (25%) and trisomy 12 in 7/56 (13%) patients, whereas by FISH those aberrations were detected in 16/62 (26%) and in 10/62 (16%) cases, respectively (P<0.0001, P=0.91 and P<0.0001, P=0.80). Deletion 17p13 was detected in 2 patients by both methods, in 1 patient only by FISH and in 1 patient only by CC-DSP30. *Conclusions.* Both CC-DSP30 and FISH methods have similar effectiveness in del(11)(q22) and trisomy 12 detection. In case of del(13)(q14) the strength of agreement between the results obtained by both methods was poor, because this deletion is often cryptic and escapes detection by CC analysis. Additionally, CC-DSP30 allows to detect other chromosomal aberrations, especially in patients with sole del(13)(q14), that can change the good prognosis associated with this abnormality.

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SECONDARY 8Q24/C-MYC TRANSLOCATIONS IN B-CELL LYMPHOMAS

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Background. c-MYC translocations are cytogenetic markers of Burkitt lymphoma (BL), with the t(8;14)(q24;q32) occurring in 80% and t(2;8)(p12;q24) or t(8;22)(q24;q11) in 20% of cases. However, c-MYC translocations with Ig or non-Ig partners also have been reported in rare cases of other B-cell neoplasms. Aim. To define clinical, morphologic, immunologic and cytogenetic features of B-cell lymphomas carrying secondary 8q24/c-MYC translocations. *Methods.* Between 2000 and 2009 in Russian Hematology Research Center c-MYC translocations were revealed in 91 cases of B-cell neoplasms by chromosomal banding or interphase fluorescence *in situ* hybridization (FISH). FISH was performed using an LSI c-MYC dual-color breakapart rearrangement probe and/or LSI c-MYC/IGH dual-color dual-fusion translocation probe (Vysis). An LSI BCL-2 and/or LSI BCL-6 dual-color breakapart rearrangement probes (Vysis) were used in 4 cases. Based on multidisciplinary diagnostic approach 84 cases were diagnosed as BL, and 7 cases as other B-cell lymphomas: 1 case as follicular lymphoma (FL), 3 cases as diffuse large B-cell lymphoma (DLBCL), 1 case as mantle cell lymphoma (MCL) and 2 cases as MGUS and multiply myeloma (MM)). In this study we analyze clinical and immunohistological data of the latter seven cases (Table1). *Results.* Aberrations involving 8q24/c-MYC were found in 3 DLBCL cases at diagnosis (#2-4). Patient #1 have had a 9-year history of t(14;18)-positive FL, t(8;14)(q24;q32) was identified in relapse with clinical features consistent with ALL. Case #5 was presented as CD10+ blastoid relapse after partial response of "classic"MCL 12 months after initial diagnosis and cytogenetically have been characterized as tetraploid complex karyotype with t(11;14)(q13;q32) and t(8q24)/c-MYC with undefined partner. In one MM patient (#7) c-MYC translocation was discovered in extramedullar relapse with normal bone marrow counts 2 years after diagnosis and was presented as t(8;11;14)(q24;q13;q32) in complex karyotype without 13q deletion confirmed by FISH. In patient #6 c-myc rearrangement was defined in progression/transformation of MGUS to plasmablastic leukemia (PBL) 5 years after diagnosis. Aberrations of 17/17p13 chromosome were detected in 2 cases of relapse/progression (#1 and #6) and in one primary tumor (#4). Histologic and cytologic features of four cases (DLBCL and MCL) were consistent with Burkitt-like lymphoma, plasmoblastic leukemia (PBL) in patient #6, one case as extramedullary transformed plasmocytoma (#7). In one case bone marrow histology was not evaluable (#1). Ki-67 was ~ 100% in cases #2-4 and #7, 42% in MCL patient and was not explored in cases #1 and 6. Bcl-2 was positive in cases #1-3(with t(14;18)(q32;q21)) and #5(with t(11;14)(q13;q32)). Case #4 was positive for BCL6 protein with 3q27 rearrangement. The clinical course of all patients was characterized by rapid progression, advanced stage, high LDH level and extranodal involvement. The median OS was 4 months (range <1-9 months) irrespective of treatment intensity. *Summary/Conclusions.* B-cell lymphomas with secondary c-myc rearrangements are highly aggressive chemoresistant neoplasms with some morphologic features of BL (cytoplasmic basophilia and vacuolization, "starry sky"pattern), frequent extranodal involvement, aberrant CD10 expression (case#5) and very short overall survival. Of interest, in our study secondary c-MYC translocations occurred more often with non-Ig partners.

Table 1.

Table 1. Clinical, cytogenetic features and survival of patients with secondary 8q24/c-MYC rearrangements.

Pt	Age/ Sex	Primary Diagnosis	FISH	Time of revelation	Survival (months)
1	79/F	FL	t(14;18)+/c-myc+	Relapse	2, DOD
2	37/M	DLBCL	t(14;18)+/c-myc+	Initial	4, DOD
3	43/F	DLBCL	t(14;18)+/c-myc+	Initial	<1, DOD
4	50/F	DLBCL	Bcl6+/c-myc+	Initial	8, DOD
5	75/M	MCL	t(11;14)+/c-myc+	Relapse	9, DOD
6	65/F	MGUS	c-myc+/del13q+	Progression/ transformation	3, DOD
7	58/M	MM	t(11;14)+/c-myc+/ del13q-	Relapse	1+ alive

DOD – died of disease.

1738

A COMBINED ANALYSIS OF CYTOGENETIC AND CLINICAL PROGNOSTIC MARKERS IN GREEK PATIENTS WITH PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

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Background. Clonal recurrent chromosome abnormalities in pediatric ALL are the hallmark of the disease and are routinely used in the pediatric setting to assist patient management in terms of diagnosis, prognosis, disease monitoring, and risk stratification. *Aims.* To correlate the cytogenetic data (karyotype and FISH) with clinical prognostic markers (age, sex, immunophenotype and white blood cell count (WBC)) in Greek patients with pediatric ALL at diagnosis. *Methods.* 85 children with ALL were included in the study, 52 males and 33 females (ratio 1.6:1), with a median age of 5 years (range 1-17). The median WBC was 10,120/μL (range 200-1×10^6/μL). 77/85 (90.5%) had B-ALL (23/58 common B-ALL, 30/58 pre B-ALL, 5/58 pro B-ALL) and 8/85 (9.5%) T-ALL. Karyotypic analyses were performed at diagnosis. FISH studies were conducted using commercial probes for the TEL/AML1 and BCR/ABL fusion genes, deletions of the INK4/9p21 locus, rearrangements of the MLL/11q23 gene and trisomies of chromosomes 4, 10, 17, 21 and X. *Results.* A successful karyotypic result was achieved in 76/85 (89.4%) cases. FISH analysis was successful in all cases studied. Combined classical and molecular cytogenetic analysis revealed clonal abnormalities in 70/85 (82.3%) cases. High hyperdiploidy (51-65 chromosomes) was detected in 24/79 (30.3%) cases. The translocation t(12;21)/TEL-AML1+ was detected in 18/85 (21.2%) and was accompanied by additional genetic changes in 13/18 (72%) cases. The secondary abnormalities in the TEL-AML1+ cases included: deletion of the non-rearranged TEL/12p13 gene (7/13, 53.8%), an extra

der(21)t(12;21) (5/13, 38.5%), an extra AML1/21q22 gene (5/13, 38.5%) and heterozygous deletion of the MLL/11q23 gene (2/13, 15.4%). More than one additional genetic changes were observed in 5/13 (38.5%) of these cases. Deletions of the INK4/9p21 locus was detected in 11/49 (22.4%) cases; 6/11 (54.5%) heterozygous and 5/11 (45.5%) homozygous. Other clonal abnormalities of prognostic value included MLL/11q23 gene rearrangements (3/84, 3.6%), translocation t(9;22)/BCR/ABL+ (2/77, 2.6%), hypodiploidy (≤44-45 chromosomes) (3/85, 3.5%), translocation t(1;19) (2/77, 2.6%) and amplification of the AML1/21q22 gene (2/85, 2.4%). High hyperdiploidy was significantly associated with age <10 years (P=0.03), B-ALL diagnosis (P=0.05) and low WBC (<50,000/μL) (P=0.03). Deletions of the INK4/9p21 locus were associated with high WBC (≥50,000/μL) (P=0.04) and homozygous deletions showed additional association with age ≥10 years (P=0.05) and T-ALL diagnosis (P=0.02). The translocation t(9;22) showed association with T-ALL diagnosis (P=0.02) and high WBC (P=0.04). The MLL/11q23 gene rearrangements were associated with age ≥10 years (P=0.007). AML1/21q22 gene amplification was associated with age ≥10 years (P=0.03). Heterozygous deletion of the MLL/11q23 gene was associated with age ≥10 years (P=0.03) detected in TEL/AML1+ cases (P=0.006). *Summary/Conclusions.* The incidence of prognostically significant chromosome abnormalities is similar to that reported in the literature. High hyperdiploidy, a cytogenetic hallmark of good prognosis, characterizes patients with favorable clinical markers. The heterozygous deletion of the MLL/11q23 is a nonrandom secondary change in TEL-AML1+ B-ALL, found in our older group of patients. Homozygous INK4/9p21 deletion was detected in patients with unfavorable clinical features (T lineage, high WBC, age ≥10 years), suggesting that this genetic abnormality may be considered as a poor prognostic marker.

1739

CYTOGENETIC PROFILE OF 451 PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA IN GREECE

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BACKROUND: Chronic lymphocytic leukemia (CLL) is associated with recurrent cytogenetic abnormalities at diagnosis or during the course of the disease which are important prognostic indicators and may influence treatment choices. However, several chromosome abnormalities have not been completely determined yet due to the low mitotic *in vitro* activity of B-CLL cells. *Aim.* We present a conventional cytogenetic study of 451 B-CLL cases in order to define the most common chromosome abnormalities of CLL and their incidence in Greek population. *Patients and methods.* Chromosome studies were performed on unstimulated and stimulated bone marrow cells, initially with tetradecanoyl phorbol acetate (TPA) and more recently with the oligonucleotide DSP30 plus IL-2. Bone marrow samples were derived from 451 CLL patients, aged 16-88 years at diagnosis or during the course of the disease between 1998 and 2010. *Results.* Three hundred and three patients were male and 148 were female. The median age was 64.6 years. Cytogenetic analysis was successful in 421 patients (93.3%). A normal karyotype was found in 249 patients (~59%), and abnormal in 172 patients (~41%). Among the abnormal karyotypes 46 exhibited complex karyotypes (≥3 chromosome aberrations) (26.7%). and 91 karyotypes (53%) carried only one aberration. The frequencies of chromosome aberrations in abnormal karyotypes were the following: +12 in 29.1%, -Y in 15.1%, abnormal (abn) 17 in 14%, abn 14q in 11%, del(6q) in 7%, del(13q) in 7%, -X in 6.4 %, numerical abnormalities of chromosome 8 in 6.4 %, del(11q) in 4.7%, del(7q) in 4.1%, -18 in 4.1%, abn 20 in 4.1%, t(11;14) in 2.3%, traslocations of chromosome 11 other than t(11;14) in 5.2%, traslocations of chromosome 18 in 3.5% and chromosome markers in 14%. The most common abnormalities found in karyotypes as sole aberrations were +12 in 28.6%, -Y in 15.1%, add(14q) in 5.5% and del(6q), del(11q), del(13q), -X in 3.3% each. In complex karyotypes, chromosome markers were found in 43.5%, abn X in 21.7%, -17 in 19.6% and del(6q) in 17,4%. *Summary/Conclusions.* The sex ratio was 2.1M/1F, similar to that reported in the literature. Trisomy 12 was the most common abnormality. Surprisingly, -Y was the second most common abnormality, either as a disease associated aberration or as a consequence of advanced age. In addition the frequencies of add(14q) as a sole abnormality and abn X in complex karyotypes were higher compared to those in the literature. The presence of geographic heterogeneity of chromosome abnormalities could be attributed to population genetics and environmental factors or combination of two. The cytogenetic complexity of CLL emphasizes the value of conventional cytogenetics which permits an overview of all microscopically visible chromosome abnormalities.

1740

MYC AMPLIFICATION AND PRESENCE OF MICRONUCLEI IN TWO PATIENTS WITH ACUTE MYELOBLASTIC LEUKEMIA (AML)

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Background. Double minutes (dmin) observed in cytogenetic studies correspond with genic amplification of some genes like MYC or MLL. Whereas this amplification is very frequent in solid tumors, in hematological neoplasias as AML have an incidence under 1%. *Aims.* Description of two patients diagnosed of AML with presence of dmin corresponding to amplification of gene MYC. *Methods.* We present a patient 54 years old diagnosed in January of 2009 of AML with predominant monocitoid component (patient 1) and a patient 81 years old diagnosed in June of 2009 of AML with multilinial dysplasia (patient 2). Samples of bone marrow were harvested according to routine. G-banding and FISH with LSI IGH/MYC, CEP 8 Tri-color, Dual Fusion Translocation Probe (Vysis) were applied. *Results.* Patient 1 showed a karyotype 46,XY, 1~20dmin[35]/46,XY[5] and patient 2 a karyotype 47,XY,+4,~5-15dmin[20]. In the two cases, the study of FISH in interphase cells showed multiple signals of MYC (rank 1~20) confirming that dmin observed correspond to the amplification of this gene. Also, it was observed the presence of micronuclei with multiple signals of MYC gene. In addition, the study of FISH in metaphase did not show deletion of MYC gene in chromosome 8, not following therefore the theory of episome model in which previous a amplification is suggested the need of the deletion of the implied gene (Storlazzi *et al.*, 2006). Morphological features of blast cells did not correspond with AML-M1 with MYC amplification described in the literature. Also, we did not observed chromatin extrusion in the blast cells, although we observed presence of micronuclei in the FISH study. In the literature is suggested that survival could have relation with the percentage of micronuclei observed, and that dmin are related to aggressiveness of the disease and resistance to chemotherapy. In the present study patient 1 died during induction and patient 2 is still alive two months after relapse (4 months after diagnosis). *Conclusions.* Further studies are needed to establish a good correlation between percentage of micronuclei and aggressiveness of the disease and resistance to chemotherapy (Storlazzi *et al.*, 2006; Villa *et al.*, 2008). Also, it is necessary to determinate if absence of chromatin extrusion have a role in the disease.

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A CYTOGENETIC STUDY OF 403 ADULT ACUTE MYELOID LEUKEMIA PATIENTS IN GREECE

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Background. Acute myeloid leukemia (AML) remains the most common form of leukemia and the most common cause of leukemia death. Diagnostic cytogenetics is considered one of the most valuable prognostic determinants in AML while current risk-group classification, is mainly based on cytogenetics and early treatment response. *Aims.* We reviewed the cytogenetic characteristics of 403 AML patients in order to investigate the frequencies of chromosome abnormalities among Greek patients with *de novo* AML and secondary AML (s-AML) which includes therapy-related AML and AML arising from a previous Myelodysplastic syndrome. *Methods.* Chromosome studies were performed on unstimulated bone marrow cells, derived from 403 AML patients, who were ≥18 years of age at the time of diagnosis. Patients were classified according to FAB classification. *Results.* Two hundred seventeen patients were male and 186 were female (M/F:1.2/1). The median age of patients was 56.5 years (range 18-95). FAB classification was available in 198 patients with 10 patients classified as M0 (5%), 14 as M1 (7.1%), 39 as M2 (19.7%), 54 as M3 (27.3%), 48 as M4 (24.3%), 25 as M5 (12.6%), and 8 as M6 (4%). Among AML patients, 341 had *de novo* AML with a median age of 55,2 years (range 18-88) and 62 patients had s-AML with a median age of 63.2 years (range 24-95). Cytogenetic analysis was performed in 341 patients with *de novo* AML and results were obtained in 314 (92.1%). Normal karyotypes were found in 104 patients (33.1%) and abnormal in 210 (66.9%) of which 59 were com-

plex (28%). The most frequent chromosome aberrations in *de novo* AML were +8 in 14.6%, t(15;17)(q22;q11-12) in 10.2%, -7/del(7q) in 7.3%, t(8;21)(q22;q22) in 5.73%, -5/del(5q) in 5.4%, -17 in 4.8%, inv(16)(p13q22) in 4.5%, +21 in 3.8%, 11q23 abnormalities in 3.5%, +11 in 2.2%, and abnormalities of chromosome 3 in 1.9%. Cytogenetic analysis was also performed in 62 patients with s-AML and was successful in 59 patients (95.2%). Normal karyotype was found in 15 patients (25.4%) and abnormal in 44 patients (74.57%) of which 16 were complex (36.4%). The most frequent chromosome aberrations were -7/del(7q) in 25.4% of patients, +8 in 25.4%, -5/del(5q) in 15.2%, abnormalities of chromosome 3 in 13.5%, -17 in 10%, +11 in 3.4%, inv(16)(p13q22) in 3.4%, +21 in 3.4%, 11q23 abnormalities in 3.3%, t(8;21)(q22;q22) in 1.7% and t(15;17)(q22;q11-12) in 1.7%. *Summary/Conclusions.* AML is slightly more common in men than women. The median age of patients with s-AML was higher than those with *de novo* AML. The main FAB subtypes showed a distribution different to that reported in the literature, with a higher incidence of M3 followed by M4 and M2. Interestingly, the most common chromosome abnormality was +8. The spectrum of chromosome abnormalities in s-AML was similar to *de novo* AML but the frequency of abnormal and complex karyotypes as well as +8 and abnormalities of chromosome 5, 7 and 3 were much more common in s-AML. The presence of geographic heterogeneity of cytogenetic abnormalities and FAB subtypes could reveal differences in constitutional or environmental factors.

1742

DETECTION AND INCIDENCE OF MUTATIONS OF KIT, FLT3 AND NPM1 IN ACUTE MYELOID LEUKEMIA

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Background. Mutations in C-kit, FLT3 genes lead to uncontrolled proliferation of leukemic cells. Also, NPM1 is with high expression in the proliferating cells. The mutations of these genes are reported in AML cases. Several studies have shown these mutations represent a prognostic marker in AML patients. *Aims.* The purpose of this study was molecular charcacteristic of leukemia, diagnosis and frequency determination of these mutations with different subtypes of FAB in AML patients. *Methods.* Blood or Bone Marrow samples from newly diagnosed AML patients with various FAB classifications were obtained in hematologic and blood transfusion centers of Iran during 3 years (Informed consent was obtained from patients). The mutations of ITD (exon 11 and 12 and interon 11) and D835 (exon 20) of Flt3 gene in patients with acute leukaemia were studied by PCR and PCR-RFLP respectively. The resulted PCR products was electrophoresed on 8% PAGE. Exon 8 and exon 17 (D816) mutations in c-kit gene were analyzed by PCR followed by CSGE and RFLP respectively. The exon 12 of NPM1 was analysed using PCR followed by CSGE.Subsequently, PCR products of positive ITD, exon 8 of c-kit and NPM1 genes have been confirmed by sequencing techniques. *Results.* The median age of adult AML was 47 +/- 12 (range from18-75) years. There was no correlation between patients with mutation status and gender and age. A positive correlation with high presenting WBC > 20000/micl (58%) was demonstrated in flt3-ITD positive (P<0.05). In 212 adult AML, Flt3 ITD and D835 mutations occurred in 18% and 6% respectively. The highest frequency of ITD and D835 mutations (16% and 4% respectively) occurred within M3 subclasses and characterized by the t (15;17). Exon 8 mutations of c-kit were diagnosed in 1.3% of AML patients and 4.7% of patients showed D816 mutations with different findings in subtypes of AML. C-kit mutations demonstrated mostly in M2&M4 cases characterized by T8-21 and INV16 (CBF leukemia). 30% of patients with CBF leukemia showed mutation in exon 17. Mutations in exon 8 were novel and confirmed by sequencing method and documented in Gene Bank (FJ189474 and FJ177639). Out of 131 patients, 23 (17.55%) were known to have NPM1 mutation, and the presence of these mutations were confirmed using sequence technique. Concerning about higher frequency of cytogenetic abnormalities among these patients, it is not unlikely that above-stated fact has accounted for the lower frequency of NPM1 mutations in our *Results.* The highest frequency of mutations was found in subtypes of M4 (30.4%), M3 (21.7%), and M5 (13.04%). Besides, out of 23 patients with NPM1 mutation, 14 cases had mutated allele A (60.8%), 5 cases allele D (21.7%) and 4 cases allele B (17.4%). Also, 21 cases (16%) had ITD mutation which 8 cases were NPM1 positive (8/23=%35) and other 13 cases were NPM1 negative (13/108=12%). *Conclusions.* we demonstrated that the FLT3-ITD mutations are frequent molecular lesions in AML patients. The presence of ITD was associated significantly with M3 morphology with T15-17. The results of this research was in line with other reports in terms of the frequency of NPM1 gene mutations in monocytic subtypes of AML (M4, M5). High frequency of these mutations in M3 subtypes as well as allele D in all subtypes and high degree of association between occurrence of NPM1 and ITD mutations can be considered as interesting points of the *Results.*

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SEVERE AND PROGRESSIVE ACUTE DEMYELINATING ENCEPHALITIS AS THE MAIN PRESENTING MANIFESTATION OF FAMILIAL HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

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Background. Hemophagocytic lymphohistiocytosis (HLH) is a life-threatening condition of severe hyperinflammation caused by uncontrolled activated lymphocytes and histiocytes. HLH includes autosomal recessive familial forms and acquired "secondary" forms. Impaired function of natural killer (NK) cells and cytotoxic T-cells (CTL) is shared by all forms of HLH. Main clinical features are persistent fever, hepatosplenomegaly and pancytopenia. A third of patients presents variable neurological signs such as seizures, opisthotonic posture, or cranial nerve palsies. Early and severe neurological findings are frequent in patients with MUNC 13.4 gene mutations. *Aim.* We report a case of familial HLH occurred with severe and unusual neurological involvement. *Case.* A, a 3 year-old boy, was admitted to our unit since a history of prolonged and unresponsive to antibiotics fever. Physical examination and laboratory showed hepatosplenomegaly and cytopenia (Hb 9.5 g/dL, MCV 76.8 fl, N 570/ul, PLT 79000 u/L), slightly elevated inflammation indexes and LDH. Triglycerides, ferritin, transaminases, bilirubin, fibrinogen were normal. Viral and bacterial infections were excluded by serology and cultures. Normal bone marrow examination excluded leukemia: blast cell and hemophagocitosis were absent. Laboratory for autoimmunity was negative. Few days after, clinical course worsened since fast progressive neurological deficit (sensitive and motor) of lower segments with hyperreflexia, loss of sphincters control and bilateral papilledema occurred. Cerebral spinal fluid exam showed increased protein level (62 mg/dL), normal glucose level (47 mg/dL) and >900 WBC/mm^3. Brain magnetic resonance (MR) detected findings of acute diffuse demyelinating encephalomyelitis: high signal intensity on the T2-weighted images mainly involving cerebral white matter bilaterally with evidence of multiple enhancing nodules after injection of contrast material; similar findings were showed within cerebellum and pons and bulbus medullae. Lateral posterior spinal segments were also involved. Persistent generalized intractable seizures finally led to exitus. The severe neurological involvement strengthened the hypothesis of HLH. Detection of very decreased NK function confirmed HLH. Familial history of the patients was negative for consanguinity but revealed the exitus of an uncle and of a sister of A. due to a suspected cerebral neoplastic disease. Later also a niece of the patient received diagnosis of HLH overt with classical symptoms and similar neuroradiological imaging of A, even without neurologic symptoms. Since these findings, we suspected a case of familial HLH. Molecular analysis of PRF1 gene, UNC 13D gene, STX11 gene, STXBP2 gene revealed no mutation causing disease. *Conclusions.* We presented a familial HLH form characterized by early severe acute demyelinating encephalopathy caused by a genetic defect not yet discovered. HLH related neurological symptoms usually develop during disease progression. Demyelination is an unusual finding in HLH. Since early diagnosis and subsequent treatment is mandatory to improve the prognosis of HLH involving CNS, HLH has to be ruled out in patients with neurological disease of unknown origin above all in presence of fever, hepatosplenomegaly, cytopenia and NK reduced function.

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RARE GROSS DELETION IN TCIRG1 GENE IN IRANIAN FAMILY WITH INFANTILE MALIGNANT OSTEOPETROSIS

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Background. Infantile malignant osteopetrosis (OMIM: 259700) is an autosomal recessive disorder, manifests by severe osteosclerosis with-

in the first decade of life. Mutations in TCIRG1 (T-cell immune regulator 1) gene encoding osteoclast-spesific 116-kD subunit of H+-ATPase named as a3 subunit were found as the cause of infantile malignant osteopetrosis type. Recent research found that mutations in TCIRG1 gene are responsible for about 50% of patients. We found the first Iranian patient with a rare gross deletion identified in this gene. *Case presentation.* Z.A. was a 5 year old girl referred to hematology department of Dr Sheikh pediatric hospital with macrocephaly, facial dysmorphism, blindness, mental retardation and hepatosplenomegaly. Laboratory investigations revealed pancytopenia. Radiological images showed osteosclerotic changes in skull and limb. With these findings she was referred for molecular analysis of osteopetrosis disease. *Methods.* Molecular analysis was performed using RT-PCR for exon 10-19 of TCIRG1 gene followed by whole gene sequencing using an ABI 3730 capillary system automated sequencer. *Results.* The patient showed a 275bp unexpected amplified segment in PCR experiment. Sequencing of the PCR product revealed a gross deletion in exon 10-15 transcript region of TCIRG1. This deletion affected codon 389 to 518 including entire exon 11 to 13 of the gene. *Conclusion.* Various types of mutations in the TCIRG1 gene in infantile malignant osteopetrosis have been reported in different populations; however, gross deletions are reported rarely. This gross deletion of exon 11-13 in infantile malignant osteopetrosis is the first mutation reported among Iranian patients in this gene. This deletion is also the largest deletion of TCIRG1 gene reported until now.

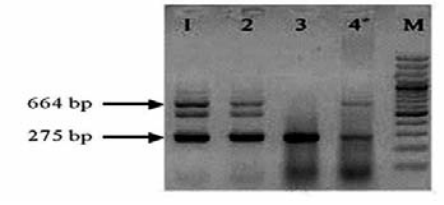

Figure.

1745

SURVIVIN MUTATION AND WILD TYPE P53 TUMOR SUPPRESSOR GENE EXPRESSION IN EGYPTIAN PATIENTS WITH ADULT ACUTE LEUKEMIA BEFORE INDUCTION OF CHEMOTHERAPY

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Background. Survivin, a member of the inhibitors of apoptosis family of proteins, is one of the most frequently upregulated transcripts in solid tumors and hematopoietic malignancies. Wild type of p53 tumor suppressor gene is involved in regulation of survivin expression in leukemic cells. *Aim of the work.* This study aims to assess the expression of wild p53 tumor suppressor gene and survivin mutation in bone marrow samples from adult acute leukemia patients before chemotherapy. *Subjects and methods.* 20 adult patients with acute myeloid leukemia and another group composed of 20 adult patients with acute lymphoblastic leukemia diagnosed by standard immunologic, morphologic and cytochemical criteria. 20 adult patients with diagnosis of hypersplenism served as non leukemic control group. DNA and RNA extraction were done manually from bone marrow samples. Survivin mutation was assessed in DNA BM samples by using syber green method and expression of wild gene p53 was assessed in RNA samples by Taqman method using Real Time PCR. *Results.* Survivin mutation was detected in 20% of bone marrow samples from patients with acute myeloid leukemia (AML) and 5% of bone marrow samples in patients with acute lymphoblastic leukemia (ALL) with absence in the third group. This result is highly significant among the three groups (P<0.0001). Wild type p53 gene expression was present in 45% of patients with AML and 25% in patients with ALL and absent in the third group P<0.001. There is a relation between p53 wild gene expression and mutation of survivin gene in the AML group. Presences of survivin mutation and/ or expression of wild p53 gene are associated with poor prognostic variables (low white blood cells, low hemoglobin and young age) in AML group.*Conclusion.* Survivin mutation is associated with expression of p53 wild gene in bone marrow samples and more prominent in AML than ALL groups. These findings may highlight the importance for screening for these genes before induction of chemotherapy.

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TRANSLOCATION (X; 10)(P10;P10) : A RARE BUT NON RANDOM CHROMOSOMAL ABNORMALITY IN ACUTE MYELOID LEUKEMIA

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Background. Sex chromosomes are infrequently involved in chromosomal aberrations in patients with hematologic malignancies. There are very few reports describing structural abnormalities of the chromosome X in acute leukemia. In most instances, the abnormality is either duplication of the q arm or deletion and translocation involving the q13 and q24 regions. *Aim.* We report herein a rare translocation t(X;10)(p10;p10) in a patient with acute myeloid leukemia (AML). A kid with 2 months and 20 days initially presented with acute leucosis. *Methods.* Cytogenetic analyses were performed by R-banding karyotype and fluorescence *in situ* hybridization (FISH) to explore the chromosomal abnormalities and confirm the diagnosis. *Results.* Bone marrow examination showed a hypercellular marrow and confirmed the diagnosis of an acute myeloid leukemia (AML4). Cytogenetic analysis detected a t(X;10)(p10;p10) in 17/27 metaphases analyzed by R-banding karyotype. Thus was confirmed by FISH analysis with whole chromosome painting (WCP) specific for chromosomes X and 10. The patient was treated with chemotherapy and a complete morphologic and cytogenetic remission was obtained after two cycles. A review of the literature and the Mitelman Database of Chromosome Aberrations in Cancer showed three previous reports of a similar translocation t(X;10) in childhood AML. The first case was a 2-year-old girl with AML-M1 and t(X; 10)(p11;p11), the second child was a 14-year-old boy with a morphologically FAB-M1-peroxidase-positive acute leukemia and T-lymphoid markers and the third patient had therapy-related AML after exposure to etoposide, which was previously given as part of ALL therapy. Translocation (X;10)(p10;p10) has also been reported in two adult patients with acute leukemia, one with acute monocytic leukemia and the other with a myeloid relapse of a bilineage leukemia. *Summary.* To our knowledge, our case is the first report of a neonatal AML4 with t(X; 10). Although the patient had an excellent early response to a salvage AML-type therapy, the prognostic significance of the t(X;10) in this setting remains unclear. Due to the rarity of this translocation, further cytogenetic and molecular biologic studies are required to elucidate the clinical and molecular significance of this unusual karyotypic finding.

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INCIDENCE OF CYTOGENETIC ABERRATIONS IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) IN LATVIA

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ALL is basically a genetic disease since most patients have genome changes causing uncontrollable proliferation and abnormal differentiation. Some genetic abnormalities in ALL patients are significant independent prognostic factors and thus provide a stratification basis for ALL. Summarizing and analysis of genetic aberrations found in children with ALL has not yet been performed in Latvia. *Objective.* A retrospective study was aimed to collect all available cytogenetic data (starting from 01.06.2006. when cytogenetics had been introduced as a routine diagnostic method for children with ALL) and to analyze their correlation with clinics and prognosis. *Materials and methods.* 38 consecutive ALL patients who had genetic analysis performed and had been treated at Oncohematology department of the Stradins University Childrens' Hospital were included. Information was collected from hospital and of E.Gulbis laboratory archives. 10% patients had T-ALL and 90% B-ALL; 63% were boys and 37% - girls; 74% were aged 1-5, 8% 6-10 and 18% were above 11; 38% of them belonged to the standard risk, 49% to medium risk and 13% to the high-risk group. At the time of the study 82% patients were alive without event, 5% were alive in relapse and 13% dead. Standard cytogenetic karyotype was obtained from 26 patients, chromosome analysis was carried out according to the standard procedure and karyotype was classified according to ISCN 2005. FISH hybridization was carried out using locus specific, centromere, telomere and full chromosomal probes according to manufacturer's instructions. FISH methods were used to determine clinically significant aberrations - hyperdiploidy, t(12;21), t(9;22), 9p21 and 11q23 rearrangements. *Results.* Genetic aberrations were found in 92% patients. High hyperdiploidy was detected in 48% patients (29% of

them without structural chromosomal changes). Statistically significant negative correlation between high hyperdiploidy and risk group was proven (73% patients with hyperdiploidy in the standard risk, 33% in the medium risk and 20% in the high risk group, P=0,02). There were no patients with proven hypodiploidy. Translocation(12;21) was found in 16% patients , pathological 9p was found in 18%, pathological 12p in10%. BCR-ABL1 and MLL-AFF1 mutations were not detected. *Conclusions.* During this research genetic aberrations were found in 92% of patients indicating an important role of genomic damage in ALL pathogenesis. High hyperdiploidy correlated with lower risk group, which corresponds to literature data on this aberration's association with favourable prognosis. Significant differences were noted between T- and B-ALL genotypes, which probably result from a difference in malignization pathogenesis. The found aberrations did not correlate with patients' sex and age. No correlation with therapy outcome was proven, although a longer follow-up would be necessary to define a prognostic relevance of the detected aberrations. The incidence of several aberrations was found to differ from literature data, requiring further study.

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RAPID AND COST EFFICIENT SCREENING OF EIGHT MOST FREQUENT FUSION GENE TRANSCRIPTS IN ACUTE LEUKEMIA USING A MULTIPLEX RT-PCR APPROACH

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Background. Recurrent chromosomal translocations are present in a considerable proportion of the acute leukemias. Many of these translocations result in the formation of specific fusion genes. The identification of particular fusion genes is of major diagnostic and prognostic importance. *Aims.* this study was designed to validate the application of an "in house"multiplex RT-PCR for detection of the eight most frequent fusion gene transcripts in acute leukemia: E2A-PBX1, TEL-AML1, AML1-ETO, PML-RARα, MLL-AF4, CBFb-MYH11, BCR-ABL, SIL-TAL. *Methods.* a multiplex RT-PCR using primers published in the literature was compared for analitical specificity and sensitivity with a commertially available kit (HemaVision). *Results.* A total of 78 patient samples at presentation were tested: 48 patients with Acute Myeloid Leukemia (AML) and 30 patients with Acute Lymphoblastic Leukemia (ALL). The fusion genes transcripts were identified in 19 cases: TEL-AML (1), BCR-ABL (4), AML-ETO (2), PML-RARα (8), MLL-AF4 (1), CBFβ-MYH11 (2) and SIL-TAL (1). These findings allowed risk stratification for 25% of AML cases and 23% of ALL cases. *Summary.* Identification of MLL-AF9 fusion gene transcript associated with detection of MLL-PTD, FLT3-ITD, FLT3-TKD and detection of mutations in NPM1 allowed evaluation of prognostic and better disease management for the most of our AML patients. This approach use a Nested-PCR method, thus being suitable for MDR monitoring with an almost identical sensitivity as HemaVision assay. Lastly, our assay exhibited good sensitivity and specificity and allowed identification of fusion gene transcripts in 24% of acute leukemia. Compared with commercially available kits like HemaVision it is considerably cheaper and for our lot of patients identified the same fusion gene transcripts as HemaVision assay.

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DOUBLE MINUTES IN ACUTE MYELOID LEUKEMIA WITH COMPLEX KARYOTYPE

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The circular, extra chromosomal amplification of specific acentric DNA fragments (double minute,dmin), are observed in 1% of acute myeloid leukaemia(AML). Most of them consist of an amplified segment from chromosome band 8q24, always including the MYC gene. Moreover in AML with complex aberrant karyotype, a loss of one TP53 allele is frequently observed. Both abnormalities generally correlate with bad prognosis. We present here a case of AML with evidence of double minutes and a complex karyotype including del 17p. The conventional cytogenetic analysis showed the t(8;11)(q24;q11) translocation concomitant with del(17)(p13.1). On the contrary, the molecular cytogenetic analysis using painting probes for chromosome 8 and 11, showed no translocation between chromosome 8 and 11, but deletion of 8q24 and addition of extra genetic material at locus 11q11. Moreover, fluorescent *in situ* hybridization (FISH) on metaphases showed the presence of dmin derived from del 8q24, carrying amplification of c-Myc. Interphase FISH evidenced the deletion of p53 locus too. This case emphasizes the relevance of FISH to clarify cytogenetic abnormalities not otherwise evident, that may contribute to give more information especially about the prognosis. Analyzing the 28 cases (27 reported and our) of AML with extrachromosomal amplification of c-Myc described so far, a possible association between double minutes, complex karyotype, and deletion of p53 is suggested. Moreover almost half of these 28 cases with dmin showed also extra cytogenetic aberrations, including loss of chromosome 17 resulting in LOH at p53 locus. The poor prognosis of these patients could be related to a possible link between dmin, p53 and complex karyotype abnormalities and not to dmin alone as previously reported. This association could in part explain the resistance to chemotherapy and high proliferation rate of AML carrying this kind of complex karyotype.

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DIFFERENTIAL DIAGNOSIS OF FANCONI'S SYNDROME: ROTHMUND-THOMSON SYNDROME

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Introduction. Rothmund-Thomson Syndrome (RTS) is a rare autosomal recessive genodermatosis caused by DNA helicase defects. It shares many clinical characteristics with Fanconi Anemia (FA).*Clinical findings.* A girl 11-year old consulted in our Hospital for FA follow-up. She had been diagnosed in her country (Colombia). On physical exam short stature, characteristic face, pronounced poikiloderma, hyperkeratosic lesions in knees, ankles and hands were remarkable. She had bilateral hypoplastic tumbs and patella were absent, with epyfisaria dysplasia and knee instability. Blood analyse was normal except for macrocytosis. Fetal haemoglobin was normal and chromosomal instability test (spontaneous and mitogen induce) were negative twice. Abdominal and cardiac ultrasounds were normal. There were no opthalmological disorders, but she had bilateral transmission deafness. She is currently on treatment with growth hormone.Clinical finding were compatible with RTS and the genetic study confirmed it: double heterozygotic mutation (transition and deletion mutation) in RECQL4 gene. *Coments.* RTS is secondary to helicase gene defects (RECQL4) and inherited in an autosomal recessive fashion. Patients are characterized by poikiloderma (cutaneous findings appear during first 6 month of life), hair loss, short stature, skeletal defects of the limbs, cataracts and a high incidence of cutaneous and noncutaneous malignancies (osteosarcoma). Conflicting results for chromosomal instability have been reported.A RTS and other similar syndromes have to be ruled out in patients who share several clinical and analytical findings with FA, but have negative chromosomal instability test.

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MYELODYSPLASTIC SYNDROME, T-LARGE GRANULAR LYMPHOCYTE LEUKEMIA AND TURNER SYNDROME: AN EXTREMELY RARE ASSOCIATION

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The incidence of Haemopoietic Malignancies associated with Turner Syndrome (TS) is extremely rare: a review of the literature identified only 23 cases with limited clinical data reported to date. Therefore, whether monosomy-X plays an important role in leukemogenesis has not been determined yet. TS is a genetic disorder characterized by partial or complete monosomy-X. Myelodysplastic Syndromes (MDS) represent a heterogeneous group of myeloid neoplasms characterized by abnormal myelopoiesis and genetic instability. T-Large Granular Lymphocyte (T-LGL) Leukemias are a well-described group of indolent neoplasms thought to derive from chronic antigen stimulation in some cases. We report a unique case of TS associated with MDS and T-LGL Leukemia: a 48-years-old Caucasian female with history of frequent respiratory infections since childhood, hepatic cholestasis, glaucoma and primary amenorrhea was admitted to the hospital for investigation of progressive neutropenia and macrocytic anemia (not nutrition-

al). The hematologic investigations revealed Hb 10.7, MCV 101, anisocytosis, reticulocytosis (2.4%), 1200 neuthophils, 5700 lymphocytes without B clonality by flow cytometry and 98% $CD2^+$ $CD3^+$ TCR alfa/beta$^+$ $CD7^+$ $CD57^+$ $CD94^+$ $CD94^+$ $CD45RA^+$. Bone marrow morphology showed dyserythropoiesis, internuclear bridging, binucleated erythroblasts, dysgranulopoiesis, no ringed sideroblasts; abnormal flow cytometric myeloid differentiation. Bone marrow trephine biopsy was compatible with MDS. Constitutional karyotype with 22 metaphases showed absence of one X chromosome in all mitosis (45,X0). In this case, the diagnosis of TS was concurrent with the diagnosis of MDS and T-LGL Leukemia because the patient omitted some anamnesis data and she missed the hospital visits since adolescent age. At the moment, the patient remains clinically stable since 5 years ago, without any treatment. The prognosis of TS patients suffering Haemopoietic Malignancies is still unknown and, perhaps, these associations may be more frequent than previously recognized. More case reporting is needed to determine the role of monosomy-X in leukemogenesis.

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GENOTYPIC HETEROGENEITY OF ALPHA-THALASSEMIA IN ROMANIAN PATIENTS

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Alpha-thalassemia is a hereditary hemoglobin disorder caused by defects in the alpha-globin genes. The majority of the mutations involved in alpha-thalassemia are extended alpha globin gene deletions. The purpose of our work was to implement a molecular diagnosis approach for patients with suspicious diagnosis of alpha-thalassemia. Patients with modified levels of cell blood count (RBC, MCV, MCH), levels of HbA2 1-2.9% and excluding other causes of hypochromic anemia were tested for the most common alpha-thalassemia deletions. DNA samples extracted from peripheral blood of all 43 subjects included in the study were genotyped using the GAP-PCR molecular method. Our results showed ten patients with modifications in the alpha-globin genes, as following: three patients are carriers of the 3.7kb deletion and in four patients was identified the MED I deletion. An interesting finding was the identification of alpha triplicated status ($\alpha\alpha\alpha$=anti 3.7kb) in 3 patients. In one of these, alpha triplicated status is associated with cd 8 (-AA) β-thalassemia mutation resulting in a thalassemia intermedia phenotype. In conclusion, our study shows that Mediterranean alpha-thalassemia deletions -3.7 kb and MED I are quite frequently found in alpha-thalassemia cases from our country and it is the first report about alpha-thalassemia in Romania. This approach could be a very efficient application in prenatal diagnosis for a thalassemia prevention programme in our country.

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CO-INHERITANCE OF HB KNOSSOS AND CD 39 MUTATION CAUSES A BETA-THALASSEMIA MAJOR PHENOTYPE IN A YOUNG PATIENT

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In the frame of beta-thalassemia mutation screening study, we investigated the first Romanian patient with thalassemia major due to compound heterozygosity for Hb Knossos and cd 39 (C-T) mutation. Hb Knossos (cd 27 [G-T]) is characterized by reduced synthesis and by interaction with beta-thalassemia, in which the double heterozygotes display typical features of thalassemia intermedia. Here we report the first case of Hb Knossos in our country. Molecular analysis of the mutations in the β-globin gene has been performed using the PCR based *Methods.* DGGE, ARMS-PCR and PCR-RFLP. Direct DNA sequencing confirmed that the propositus is compound heterozygous for Hb Knossos (cd 27 GCC-TCC) and cd 39 (C-T) mutation. Hb Knossos is a variant with a single base substitution causing amino acid replacement and alternative splicing of precursor beta-messenger RNA by activating cryptic donor sites in the exon I. CAG-TAG substitution at codon 39 in beta-globin gene changes codon 39 into a stop codon terminating translation. Hb Knossos displays a slightly decreased oxygen affinity; this factor may compensate in part for the severe anemia of the double heterozygotes. In our case co-inheritance with severe β^0 mutation causes the beta-thalassemia major phenotype and this is important for genetic counseling.

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VNTR POLYMORPHISMS IN TPMT GENE PROMOTER: POTENTIAL TOOL FOR THIOPURINE-GUIDED THERAPY

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Background. Thiopurine S-methyltransferase (TPMT; EC 2.1.1.67) is an enzyme that metabolizes immunosuppressive thiopurine medications, used in treatment of autoimmune diseases, cancer and in transplantation medicine. Its activity is polymorphic and a trimodal distribution has been demonstrated in Caucasians (low, intermediate and high methylator groups). It has been shown that certain TPMT gene polymorphisms affect TPMT enzyme activity. TPMT gene promoter contains a variable number of three types of GC-rich tandem repeats, A, B and C, ranging from 3 to 9 in length, arranged in a specific manner (AnBmC). *Aims.* We analyzed the TPMT promoter tandem repeat distribution of alleles and genotypes in Serbian population (73 childhood acute lymphoblastic leukemia (ALL) patients, 31 adult myeloid leukemia (AML) patients and 50 blood donors). We also investigated the influence of number and type of promoter tandem repeats on transcription of TPMT gene. *Methods.* We performed functional analysis of the TPMT gene promoter and its upstream regulatory region, analyzed the interaction of the A, B and C tandem repeats with transcription factors by electromobility shift assays (EMSA) and investigated the influence of 6-mercaptopurine (6-MP) on TPMT gene transcription. *Results.* We have detected 11 different types of VNTRs in the TPMT gene promoter in the Serbian population. Number of repeats ranged from 4 to 8. Within the TPMT promoters containing the same number of tandem repeats, different architecture of VNTRs has been determined, (AnBmC, where n ranged from 1-5 and m ranged from 1-6). We have determined 17 different TPMT VNTR genotypes in Serbian population. Our data show that the distribution patterns of VNTR alleles do not significantly differ among ALL and AML patients and normal individuals. Functional assays revealed that TPMT promoter with the highest activity was the one with VNTR*4b type (AB2C). Promoters with 5, 6 and 7 VNTR alleles all had successively lower activities. VNTR*8 activity was two times higher than activity of VNTR*7 types. We found differences in activity between the constructs containing the same number, but different type of tandem repeats. The most prominent difference was observed between VNTR*4 variants (A2BC and AB2C). Also, we demonstrated that the A repeat has a negative effect in TPMT gene transcription and that a positive regulatory element, identified immediately upstream to the VNTR region of the TPMT gene promoter, is indispensable for TPMT gene transcription. TPMT gene promoter demonstrated a specific response in cells treated with 6-MP (10 μM) in a VNTR-specific manner. EMSA indicated that the Sp1 and Sp3 transcription factors bind to the VNTR repeats. *Conclusions.* Our data indicated that both the number and type of VNTRs as well as the upstream regulatory region of the TPMT gene promoter determines the overall level of TPMT gene transcription. Our results showed that VNTR geno-

types that contribute to low-methylator TPMT phenotype, account for one third of TPMT VNTR genotypes in Serbian population. For that reason promoter tandem repeats could be considered as candidate pharmacogenetic marker. Further investigation will confirm if it could be of clinical importance for individualizing thiopurine therapy.

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ACETYLSALICYLIC ACID (ASA) NON RESPONDER STATUS IN PATIENTS WITH CORONARY AND CEREBROVASCULAR DISEASE

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Background. Platelets play an important role in the pathogenesis of arterial thrombosis. Inhibition of platelet functions is an established intervention for the preventions of thrombosis events in patients with CAD, who were taking low dose ASA therapy as an antiplatet agent. The purpose of the present study was to determine the concept of ASA resistance on non responsiveness, analyzing the effect of low dose ASA therapy in patients wit CAD. *Methods.* Platelet function was evaluated using a plated function analyzer PFA-100 by determining the time to occlusion (close time CT) of an aperture in a membrane, coated with Coll/ADP or Coll/EPI as citrated whole blood flows under high shear stress conditions CT ranges with Coll/EPI cartridge was 68-167s (max. value>300s) and Coll/ADP 68-122s (max value>300s). Our study was conducted on74 patients, all of them had a chronical (6-60 months) low dose ASA therapy (100 mg/day). Tests were performed 24 hours after the last dose of ASA within a range of 30-120 minutes after blood collection. *Results.* From 74 CAD patients with chronic low dose ASA therapy 58 (78%) showed an adequate response of ASA (Coll/EPI > 167s) whereas 16 (22%) of these patients had confirmed poor response to ASA (Coll/EPI <167). Coll/ADP - CT was not significantly different. A dosage increase of 200 mg/day ASA for two months led to prolonged Coll/EPI - CT > 167s as well in 10 (62.5%) of poor responders. Patients without adequate response to ASA were treated with another platelet inhibitor or a combined medication. *Conclusion.* Our previous results, acquired by PFA-100, with Coll/EPI cartridges 24 hours after the last ASA intake, indicated that a lot of CAD patients seem to be non-responder to ASA therapy. Our study has showed: - importance of monitoring the efficacy of ASA therapy, - identification of the patients with a poor response platelet inhibition inducted by ASA therapy, - need to search an individually adapted ASA dosage or treatment with other platelet inhibitors.

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ROLE OF REAL-TIME QUANTITATIVE RT-PCR TO MEASURE MDR1 GENE OVEREXPRESSION AND ITS ROLE IN IMATINIB RESISTANCE CHRONIC MYELOID LEUKEMIA PATIENTS

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Background. Different mechanisms could sustain Imatinib resistance, including overexpression of MDR1, a gene already known to be responsible for multidrug resistance and poor prognosis in other hematologic malignancies. MDR1 overexpression may be considered as an important clinical mechanism in the diversity of resistance development in imatinib monotherapy in CML patients. *Aims.* - To develop sensitive detection of MDR1 gene in the peripheral blood mononuclear cells from in CML patients receiving imatinib. - To set up real-time quantitative RT-PCR technique to measure MDR1 gene overexpression and its clinical significance in the patients with chronic myeloid leukemia treated with imatinib. *Methods.* A cohort of 75 patients with chronic myeloid leukemia enrolled in AIIMS (2005-2009) were included in this study. Total RNA was isolated from the peripheral blood mononuclear cells from CML patients receiving imatinib. The RNA was quantified spectrophotometrically at 260 nm (ND-1000, Nanodrop technologies). First strand cDNA's were synthesized from 1ug total RNA .Real-time quantitative RT-PCR for MDR1 was performed with SYBR Green Supermix. The primers used for MDR1 and beta-actin were selected based on specificity and efficiency by qPCR analysis of a dilutions series of pooled cDNA at a temperature gradient for primer-annealing and subsequent melting curve analysis,agarose gel-electrophoresis and nucleotide sequence analysis. All patients were monitored for hematologic and molecular responses, time to progression, survival and toxicity. *Results.* Thirty of 75 imatinib resistant CML patients had high levels of MDR1 expression. The higher expression of MDR1 levels were also reported in higher age group ,≥40 years (60% *vs.* 40%; P<0. 05), and leukocyte counts ≤50×10^9/L (70% *vs.* 46%; P<0.01) but the lower expression of MDR1 was reported in the CML patients with p-loop mutations. The defined cut off for copy numbers of MDR1 transcripts corresponded with the degree of biological resistance in the peripheral blood mononuclear cells of relapsed CML patients. *Conclusion.* The molecular technology developed has a prognostic importance. Real-time quantitative RT-PCR technique using Sybre green proved economical, rapid and sensitive. MDR1 expression may play an important role in Imatinib resistance in CML patients. These results suggest the efficacy of this quantitative analysis of MDR1 expression by Sybre green to predict clinical drug resistance in CML patients receiving imatinib.

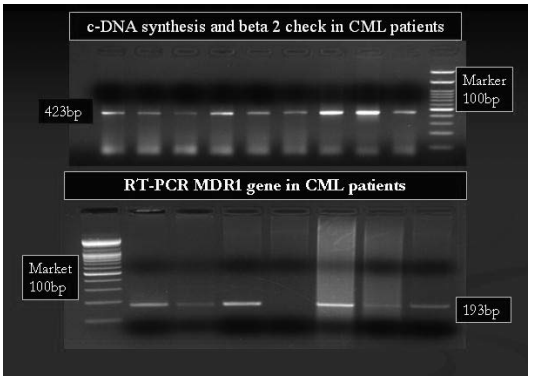

Figure. RT PCR for MDR1.

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THE FIRST HYPERTRIGLYCERYDEMIA CASE REPORT WHILE MOBILIZATION OF AUTOLOGOUS PERIPHERAL BLOOD HEMATOPOIETIC CELL WITH FILGRASTIM

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Autologous peripheral blood hematopoietic cell transplantation is one of the treatment alternative for Hodgkin lymphoma. While mobilization regimen of the autologous peripheral blood hematopoietic cell transplantation the group of myeloid growth factors are use. Filgrastim is the most used drug for mobilization of hematopoietic cells. Some side effects of Filgrastim are fever, splenomegaly, bone pain, central nervous system effects etc. There was no report of hypertriglyceridemia after exposure of Filgrastim. We present a severe hypertiglyceridemia case report after exposure of Filgrastim for mobilization of autologous peripheral blood hematopoietic cell. 40 year old man treated for stage IIA nodular sclerosis Hodgkin lymphoma. Patient takes Bornaprine 4 mg/d, Escitalopram 20 mg/d treatment for anxiety disorder. He had no metabolic disease on routine controls. Triglyceride level was 200 mg/dL on routine examination. After 3 years with remission he relapsed with cervical and mediastinal lymphadenopathy. After treatment with 3 courses of DHAP (Dexamethasone, cisplatin, high-dose cytarabine) chemotherapy regimen, patient reviewed as complete remission. Autologous peripheral blood hematopoietic cell transplantation treatment was considered. Before Filgrastim treatment triglyceride level was 230 mg/dL. For mobilization of autologous peripheral blood hematopoietic cell 10 μg/kg/day Filgrastim was used. While fourth day of treatment chilous blood sample was seen on routine blood sample and 1180 mg/dL hypertriglyceridemia, cholesterol 235 mg/dL was seen. The control study was done 2 days later and 650 mg/dL hypertrigliceridemia was seen. The patient received no additional or lipid lowering therapy. After 1 week exposure of the Filgrastim the triglyceride level was fall to 227 mg/dL. This is the first case that has hypertriglyceridemia after exposure of the Filgrastim. There is no data at the literature for this situation. Because of this we do not know the Filgrastim effect on the hypertriglyceridemia. But on the mechanism of the hypertrtiglyceridemia lipoprotein lipase, Apo C-II, Apo-V, Apo E or hepatic lipase deficiency has a great role. Filgrastim may have a role on the clearance or the function of these enzymes and apolypoproteins. In conclusion Filgrastim has wide range of use on mobilization of hematopoietic stem cell at healthy donors as patients. For this reason scanning of triglyceride levels must be done for patients and donors.

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MYOCARDITIS INDUCED BY ALL-TRANS RETINOIC ACID (ATRA)

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The introduction of all-trans retinoic acid (ATRA) in the therapy of acute promyelocytic leukaemia (APL) has improved the outcome of this rare hematologic malignancy. ATRA is usually well tolerated but some side effects have been described: headache, pseudotumor cerebri, nasal stuffiness, dry red skin, chapped lips, transient elevations in serum aminotransferases and bilirrubin and hypertriglyceridemia. However, the most important complications are: differentiation syndrome and hyperleukocytosis. In the literature, a few cases of myocarditis as drug toxicity are also refered. We present a case of myocarditis induced by ATRA in a patient with t(15;17) positive microgranular APL with PML-RAR alfa rearrangement and a negative cardiac history. Our patient, a 28 year old female, was treated with ATRA in combination with idarubicin and cytosine arabinoside (Ara-C) due to her high risk leukaemia (leukocytosis: 30.300/mm^3). During the first days of therapy, beyond fever the patient had volume loading, weight gain and signs of pulmonary hypertension on echocardiography. This clinic was resolved with diuretics. On second day of treatment the patient had macula hemorrhage on left eye. On day 20, the patient complained of tightness, which increased with deep breath and movements. The electrocardiogram showed ST elevations from V1 to V4. Laboratory analysis showed a minor increase of troponin (0,438 μg/L) with normal CK-MB. Echocardiography showed a pattern of myocarditis with an ejection fraction of 22%. ATRA was discontinued and she was given dexamethasone (first dose: 10 mg twice a day with decrease dose afterwards). The patient needed to be at intensive care unit during two days because she developed hypotension and oligoanuria. She was started on digitalis and dopamine therapy with a good clinic evolution although the ejection fraction was still of 11%. All serological tests for viral infections proved to be negative. The patient recovered and achieved complete remission on day 28. The echocardiographies done after that date were normal. In the following consolidations, ATRA was reintroduced, and she only had subclinic hypothyroidism in relation with ATRA. Nowadays she is receiving maintenance therapy consisting of 6-mercaptopurine and methotrexate once a week. In conlusion, we present a case of myocarditis due to ATRA who recovered completely with the use of steroids and was subsequently retreated with ATRA without recurrence of the myocarditis.

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USE OF HEMOGLOBIN RETICULOCYTE TO DETERMINE THE RESPONSE TO ERYTHROPOIETIN.

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Objectives. Determine the average value of Hbr in patients with blood diseases that are indicated in the use of EPO to treat anemia, assessing the response to this at different times of treatment, checking if a parameter predictor of response to EPO. *Patients, materials and Methods.* Patients Hospital Juan Ramón Jiménez de Huelva diagnosed in our department of hematological malignancies who had received treatment with EPO from March 2007 until the present, excluding all those who have not made any determinations. We studied a total of 31 patients (16 women and 15 men) with a mean age of 64±14 years. For conditions were: 17 SMD, 8 MM, 1 NHL and 5 LLC. Was used EPO 30000 IU / week were solicited for compassionate when necessary. The control points of treatment were conducted in the 3rd, 6th and 12th week, defined as early responses (3rd week), middle (6th week) and global (12th week). Response was considered to increase at least 1 g / dl from baseline in hemoglobin. For the analysis of the counter was used Hbr 2100 Sysmex Roche according to manufacturer's instructions and software with the quality standards currently in force. Statistical analysis was performed with SPSS 14.0 software. The significance of differences between means was analyzed with the Mann Whitney test, dichotomous variables were compared with Chi-square. The significance level was set at p value <0.05. *Results.* Patients with EPO response to early, intermediate and overall had a higher Hbr to nonresponders. The response by increasing the level of hemoglobin in patients treated with EPO was 1.2 g/dL. Answered a total of 14 patients (43%) *vs.* 17 who did not (57%). Hbr Mean baseline study patients was 32.5 pg. (being the average healthy population in our area of 36.5 pg.). In the 3rd week of treatment with EPO, patients with a Hbr> 32.5 pg., Showed a response rate of 65% *vs.* 35% in nonresponders (P>0.05). Patients with Hbr <32.5 pg. Had 20% response *vs.* 80% of nonresponders (P=0.04). Hbr Having an average of 33 pg responders., And non-responders of 26.5 pg. At the 6th week of treatment with EPO, patients with a Hbr> 32.5 pg., I present a 70% response compared to 30% in non-responders (P=0.035). Patients with Hbr <32.5 pg. Had a 32% response compared to 68% of nonresponders (P=0.045). Hbr Having an average of 36.2 pg responders., And non-responders of 29.5 pg.At the 12th week of treatment with EPO, patients with a Hbr> 32.5 pg., Introduced a 80% response *vs.* 20% in non-responders (P=0.035). Patients with Hbr <32.5 pg. Had 30% response compared with 70% of nonresponders (P=0.045). Hbr Having an average of 37.1 pg responders. And non-responders, 31 pg. *Conclusions.* In this study we evaluated the early Hbr as a parameter which rises at the time of starting treatment with EPO, making it a simple method of prediction of response in patients with hematological malignancies with this treatment.

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MICRORNA EXPRESSION PROFILING IN CUTANEOUS T CELL LYMPHOMAS. PRELIMINARY RESULTS

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Background. Primary cutaneous T cell lymphomas (CTCL) represent 70% of all cutaneous lymphomas being the most frequent subtypes the mycosis fungoides/Sézary syndrome (MF/SS) and the anaplastic large T cell lymphoma CD30$^+$ (cALCL-CD30$^+$). In this entity, few high resolution genomic studies have been performed. The recent development of new technologies in genomic analysis offers the opportunity to improve the knowledge regarding microRNA (miRNA) expression profiles in human neoplasm as well as its potential role as prognostic markers. *Aims.* Our aim was to explore the miRNA expression signatures in MF tumoral stage and cALCL-CD30$^+$ using a microarray platform. *Methods.* Twenty-two patients with MF tumor stage (MFt) and 11 patients with cALCL-CD30$^+$ were included. RNA was extracted from frozen tissues containing more than 70% of tumor cells. As controls, we used a subset of 5 inflammatory diseases (ID, n=3 erythematous lupus, n=1 bullous pemphigoid and n=1 psoriasis). MicroRNA microarrays technology was performed to explore the global miRNA expression profile using the Human miRNA microarray (V2) from Agilent Technologies. For determining differentially regulated miRNAs moderated paired t-tests were applied using LIMMA. Probes with FDR adjusted p-value below 0.05 and additionally a fold change exceeding 1.2 in absolute value were selected as the relevant ones. All statistical analyses were performed with the Bioconductor project (v2.3) in the R statistical environment (v.2.8.1) For cluster analysis, probes which had a minimum coefficient of variation of 0.05 across all samples were selected and a correlation-based hierarchical clustering based was performed. *Results.* Regarding microRNA expression, a signature composed of 41 microRNAs was found to be differentially expressed in MFt compared with ID including the miR-142, miR-200b, miR-200a, miR-429 and miR-452 (adjusted-p value <0.001). Regarding cALCL-CD30$^+$, 36 microRNAs were found to be differentially expressed compared with ID including miR-155, miR-21, miR-200b, miR-200a, miR-30a and miR-429 (adjusted-p value <0.001). The unsupervised clustering did not segregate the lymphomas into two different clear groups. *Summary.* The application of microRNA expression microarray technology has allowed to define the microRNA signature of CTCLs. Surprisingly, both types of lymphomas have shown a similar miRNA expression profile while the clinical behaviour and the genomic profile of these two entities is quite different. The exhaustively analysis in terms of target gene analysis and the correlation with clinical data is a reasonable next step for the understanding of pathogenic mechanisms of these microRNA and also the definition of biological prognostic factors. *Acknowledgements.* RTICC grant No. RD07/0020/2004 from the Ministry of Science and Innovation.

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NOVEL DELETION IN SLC19A2 GENE ASSOCIATED WITH TRMA IN A CONSANGUINEOUS NATIVE OMANI FAMILY

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Background. Thiamine-responsive megaloblastic anemia (TRMA) syndrome, a rare autosomal recessive early-onset condition, is characterized by a diagnostic obligate triad of clinical features: progressive sensorineural hearing loss, diabetes mellitus and megaloblastic anemia. Loss-of-function mutations in SLC19A2 gene [1q23.3] encoding the high affinity/low capacity thiamine transporter (THTR-1) have been found to be causal of TRMA in affected kindreds from different populations. Pharmacological doses of thiamine improve the hematologic picture and may delay the onset of diabetes mellitus or reduce the insulin requirement but the hearing loss seems to be irreversible or may require thiamine supplementation early in fetal/neonatal life. Here, we report a novel homozygous deletion in the SLC19A2 gene in large consanguineous Omani family with six clinically recognized TRMA cases, two of which died early in infancy. *Aim.* To define the underlying molecular mechanism of apparent TRMA syndrome, in a child referred with megaloblastic anemia, diabetes mellitus and sensory neural hearing loss. *Methods.* Genomic DNA, isolated from peripheral blood of the patient and her family, was mapped using two microsatellite markers D1S1569 and TMG86 and the alleles were separated on an ABI 3100 automated DNA sequencer and genotypes were analyzed with ABI GeneMapper software 3.0.All the 6 exons of the SLC19A2 gene were PCR -amplified and cycle-sequenced utilizing the BigDye Terminator sequencing kit v3.1. To define the deletion breakpoint region, PCR reactions were carried out using primer pairs located in intron 3 and 3'-untranslated region with Expand Long Template PCR kit. A simple and rapid multiplex PCR strategy was designed for diagnostic purposes. *Results.* Haplotype analyses revealed that the proband was homozygous by descent for the two microsatellite markers, consistent with likely association of the disease phenotype with the SLC19A2 gene mutation. (Figure A) The PCR amplification of all exons of the SLC19A2 gene revealed absence of PCR product for exons 4, 5 and 6. The long range PCR identified an unusual 1.9-kb PCR- product, which were present only in the proband (homozygous) and the parents (heterozygous). Direct sequencing of this 1.9kb product defined the deletion size (5224bp) and the breakpoint region. Based on these data a simple multiplex PCR strategy was designed and used to screen for the novel deletion in the remaining members of the family especially to define the carrier status (Figure B). *Summary and conclusions.* We have identified a novel intragenic deletion of 5224bp encompassing exons 4 to 6 in the SLC19A2 gene in our proband and three other late- referred patients from a consanguineous native Omani family as causal of TRMA syndrome. The predicted THTR1 protein which results from this deletion is truncated by the c-terminal 154 amino acids with consequent absence of the seventh and eighth transmembrane helical regions. This very likely causes intracellular retention of the mutated protein with absent cell surface expression. A simple, rapid and cost-effective diagnostic strategy was established in order to simplify the mutational screening in the large extended family members.

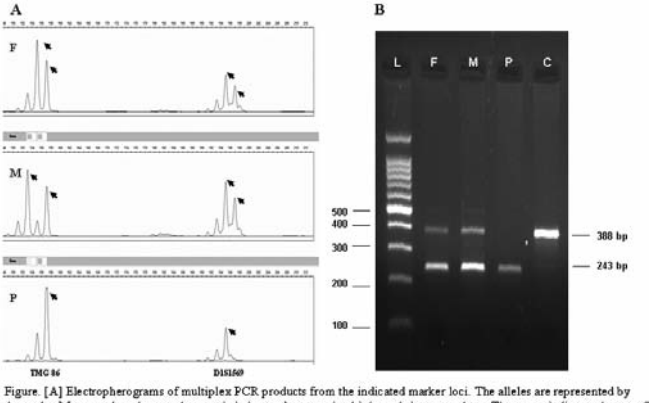

Figure. [A] Electropherograms of multiplex PCR products from the indicated marker loci. The alleles are represented by the peaks. Many markers have a characteristic 'stutter' pattern (multiple peaks), as seen here. The arrow indicates the actuall allele peak. [B] Molecular analysis of the SLC19A2 deletion. Multiplex PCR reaction was performed to amplify control (388bp) and deleted fragments (243bp).
L, (100bp ladder), F (Father), M (Mother), P (patient), and C (Control).

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SEROLOGY IS A SENSITIVE TOOL FOR DETERMINING RECIPIENT'S IMMUNE CELLS PERSISTENCE AFTER ABO-MISMATCHED HAEMATOPOIETIC STEM CELLS TRANSPLANTATION

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Background. ABO histo-blood groups are a system of strong immunogenic antigens on red cells involved not only in transfusion but also in transplants. Contrariwise to solid organ transplantation, haematopoietic stem cells transplantation (HSCT) is generally accepted also between pairs ABO-mismatched. The presence of post-transplant ABO chimera is one of the most important indicator for the residue of recipient's haematopoietic and immune cells. *Aims.* Immunology, immunogenetics and molecular genetics are able to provide, nowadays, wide evidences about chimerism after HSCT. The information they bring, in determining host's maintenance of immunological function, could be quite different. This prompted us to compare three techniques: serology, erythrogenomics and molecular analysis of microsatellite regions, in order to establish a hierarchy of accuracy in determining donor/recipient chimerism after HSCT. *Methods.* We analyzed three patients with haematological malignancies who underwent HSCT ABO-mismatched; data regarding the disease, recipient's and donor's blood group before transplant and the clinical outcomes are shown in the table. Patients 1 and 2 were characterized by a minor ABO incompatibility, while patient 3 had a major one. In pre- and post-HSCT samples, red blood cells phenotype was determined either with classical serological techniques and with molecular typing through PCR-SSP. All cases were tested for chimerism analysing selected polimorphic short tandem repeats (STR). *Results.* Different results were obtained dealing with the technique used. In particular, proteomic analysis showed the persistence of recipient's blood group post-HSCT, whereas ABO molecular typing underlined the presence of a full donor genotype. The determination of post-transplant chimerism throughout the study of microsatellite regions, highlighted in patient 3 the occurrence of 5% of recipient's cells and in patient 1 and 2 a full donor genotype. All patients are alive, however the first relapsed, the second one developed a cGVHD and the last is still requiring transfusional support. *Conclusions.* Even though ABO incompatibility is not considered a contraindication in HSCT, its clinical impact and management still remains a challenge for haematologists and transfusion physicians. Our observations, obtained from selected models, evidence the disparity between data obtained by classical serology and genomics. Interestingly, the former technique seems to be the most sensitive tool to detect host's residual immune cells after ABO-mismatched HSCT, while the erythrogenomic does not seem to have sufficient sensitivity to detect the presence of recipient's remaining cells if they are persistent in little amounts. The analysis of STR seems to be a good method to detect chimerism only in case of major ABO incompatibility.

Table. Blood groups and chimerism in ABO mismatched HSCT.

		Patient pre-HSCT			
	Disease	Serology	Erythrogenomics		
Patient 1	APL	A	A^2O^2		
Patient 2	MM	A	nt		
Patient 3	AML	O	O^1O^{1v}		
		Donor			
	Sibling/MUD	Serology	Erythrogenomics		
Patient 1	sibling	O	nt		
Patient 2	sibling	O	nt		
Patient 3	MUD	AB	A^1B^1		
		Post-HSCT analysis			
	Serology	Erythrogenomics	Microsatellites	Follow-up	
Patient 1	direct test: A indirect test: anti-B	O^1O^2	full donor (8 test)	relapse	
Patient 2	direct test: A indirect test: anti-B	O^1O^2	full donor (4 test)	cGVHD	
Patient 3	direct test: O indirect test: anti-A and anti-B	A^1B^1	5% recipient (8 test)	needing tranfusion of GRC and PLT	

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THE SURVEILLANCE OF ANTI-CANCER MECHANISM OF CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE II (CAMKII) INHIBITOR IN MULTIPLE MYELOMA CELL LINE

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Background. The study of novel drugs has been sparkled by the advent of new agents showing anti-cancer effect against multiple myeloma by interfering with various molecular pathways of proliferation and survival. Calcium/calmodulin-dependent protein kinase II (CaMKII) is a protein distributed mainly in central nervous system, known to get involved with long-term potentiation memory. neuronal cell development, cellular immunity and signal pathway of cardiac muscle cell. Several studies of CaMKII on cancer were reported regarding the c-FLIP expression regulation in apoptosis, replication factor C dependent cell cycle regulation, and regulation of Raf-1/Mek/Erk or Wnt/β-catenin signaling pathway. *Aims.* As there has been so far no study concerning CaMKII in multiple myeloma, this study was aimed to investigate the role of CaMKII, by using KN-93, a specific inhibitor of CaMKII. *Methods.* Cell viability test was done in multiple myeloma cell lines including RPMI8226, U266, ARH-77 and NCI-H929 after treatment with KN-93. Microarray technique with NimbleGen Human Whole Oligo 12-plex chip was done after KN-93 treatment on U266 cell line to evaluate the change of mRNA expression. The gene expression profile was analyzed in terms of the proliferation or survival signals of cancer cell. *Results.* The treatment with 10μM of KN-93 resulted in more than 50% of cell death rate in all multiple myeloma cell lines. Gene expression was variable in terms of proliferation or survival signal on analysis of the NimbleGen Human Whole Oligo 12-plex chip results. *Conclusions.* CaMKII is one of the important factors controlling proliferation and survival of cancer cell, and various cell signal transduction pathways. KN-93 has a definite anti-cancer effect on multiple myeloma cell line.

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CYTOKINE GENE POLYMORPHISMS AND RESPONSE TO IMMUNOSUPPRESSIVE TREATMENT OF ACQUIRED APLASTIC ANEMIA IN KOREA

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Background. The responsiveness of acquired aplastic anemia (AA) to immunosuppressive therapy (IST) is the important clinical evidence supporting an underlying immune pathophysiology. Immune-mediated marrow failure are mediated by cytokines which induce apoptosis in hematopoietic progenitor cells. *Aims.*

To evaluate whether single nucleotide polymorphisms (SNPs) of cytokine genes are related to the risk of AA and the response to IST with antithymocyte globulin and cyclosporin. *Methods.* We tested for an association between SNPs in the gene encoding interferon-gamma (IFN-γ; rs7139169, rs2069705, rs2430561), tumor necrosis factor-alpha (TNF-α; rs1799724, rs1799964, rs1800629, rs1800630), transforming growth factor-beta (TGF-β; rs1800469, rs1800470) and FAS (rs1800682) and the risk of AA in 80 adult patients and 84 age and sex matched healthy control in Korea. We then examined the relationship between these SNPs and the response to IST in 44 patients who received IST. *Results.* The minor allele (T) of a variant in the promoter region of IFN-γ (rs7139169) was negatively associated with the risk of AA (odds ratio, 0.3; 95% CI, 0.12 to 0.75; P=0.01), whereas this allele showed no association with the response to IST (odds ratio, 0.65; 95% CI, 0.15 to 2.86; P=0.57). Other two SNPs in IFN-γ gene, four SNPs in TNF-α, two SNPs in TGF-β and one SNP in FAS showed no significant association with the risk of AA and the response to IST. *Summary/Conclusions.* Our results indicate that the minor allele (T) of a SNP in IFN-gamma (rs7139169) among 10 candidate SNPs is significantly associated with the reduced risk of AA in adults. However, all of these SNPs have no apparent impact on susceptibility to IST of AA patients.

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ELA2 AND HAX1 GENE MUTATIONS IN IRANIAN CONGENITAL NEUTROPENIC PATIENTS

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Severe congenital neutropenia (SCN) is a rare primary immunodeficiency. Typically, affected children are recognized in early infancy with recurrent bacterial infections, low absolute neutrophil counts (mostly less than 500/μL) and also, maturation arrest at the promyelocyte-myelocyte stage in their bone marrow. Different genes are found to be associated to SCN, including ELA2, HAX1, WAS, GFI1, G-CSFR and G6PC3. *Aim.* The aim of this study is to determine the new mutations of ELA2 and HAX1 in Iranian patients with severe congenital neutropenia referred to Immunology, Asthma and Allergy Research Institute from 2008 to 2010. *Methods.* Patients with persistent severe neutropenia, recurrent infection and maturation arrest at promyelocyte-myelocyte stage in their bone marrow entered in this study. Genomic DNA of the patients and their parents was extracted from peripheral blood sample (3 ml whole blood), and all 5 exons of ELA2 and 7 exons of HAX1 genes and their respected flanking regions were amplified by polymerase chain reaction and subjected to direct sequencing. *Results.* Sequence analyses of ELA2 and HAX1 genes in 15 patients had revealed 6 cases of homozygous HAX1 mutation in exon 2, including 5 W44X mutations and one W59X mutation. One patient was heterozygous for ELA2 mutation in exon5 (P257L), and one showed sporadic ELA2 mutation in exon3 (V98L, V101L). The other 7 patients had no mutation in ELA2 and HAX1. *Conclusions.* It can be concluded that ELA2 and HAX1 genes are more common in our patients. Despite previous reports, we found HAX1 mutation more than ELA2 mutation in the patients. Based on our results, so far, no new mutations in ELA2 and HAX1 observed in Iranian neutropenic patients included in this study. Further studies to find other responsible genes for neutropenia, are under investigation in our lab.

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CERAMIDES REGULATE RESVERATROL-INDUCED APOPTOSIS IN HUMAN ACUTE PROMYELOCYTIC AND CHRONIC MYELOID LEUKEMIA CELLS

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Purpose. Resveratrol, an important phytoalexin in many plants, has been reported to have cytotoxic effects on several types of cancer. Ceramide is a bioactive sphingolipid that regulates many signaling pathways including cell growth and proliferation, senescence and quiescence, apoptosis, and cell cycle. Ceramides are generated by LASS gene family (LASS1-6) and they are converted to antiapoptotic molecules, glucosylceramide and sphingosine-1-phosphate by glucosylceramide synthase (GCS) and sphingosine kinase-1 (SK-1) genes, respectively. *Aims.* In this study, we examined possible cytotoxic effects of resveratrol on acute and chronic myeloid leukemia cells and determined the roles of ceramide metabolizing genes in resveratrol-induced apoptosis in addition to investigate the possibility of increasing the sensitivity of HL60, acute promyelocytic leukemia (APL) and K562, chronic myeloid leukemia (CML), cells to resveratrol by manipulating sphingolipids. *Methods.* Antiproliferative effects of resveratrol, ceramide mimetics, GCS and SK-1 inhibitors were determined by XTT cell proliferation assay. Changes in caspase-3 enzyme activity were determined by caspase-3 colorimetric assay. The mitochondrial membrane potential (MMP) was measured by JC-1 MMP detection kit. Expression levels of ceramide metabolizing genes were examined by RT-PCR. *Results.* There were synergistic cytotoxic and apoptotic effects of resveratrol in combination with ceramide analogs and inhibitors of ceramide converting agents as compared to any agent alone as determined by XTT cell proliferation and apoptotic assays in both HL60 and K562 cells. More importantly, gene expression analyses revealed that there were significant increases in the expression levels of LASS genes and decreases in expression levels of GCS and SK-1 genes in HL60 and K562 cells in response to

increasing concentrations of resveratrol in a dose-dependent manner. *Summary/Conclusion.* Our data, in total, showed for the first time that resveratrol induces apoptosis in APL and CML cells through increasing ceramide generation and decreasing conversion of apoptotic ceramide to antiapoptotic glucosylceramide and sphingosine-1-phosphate.

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THE MECHANISM UNDERLYING BIOLOGICAL EFFECT OF VALPROIC ACID IN TARGETED THERAPY OF AML1/ETO POSITIVE CELLS

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Background. In t(8;21) acute myeloid leukaemia (AML), leukemogenesis is supposed to be promoted by interference with expression of AML1 target genes. Repressor complex associated with AML1/ETO fusion protein recruits class I histone deacetylases (HDAC). Valproic acid (VPA) was found to have an extensive effect on AML blasts, via inhibition of class I HDAC. It was shown previously that VPA treatment disrupts the AML1/ETO-HDAC1 complex from AML1 promoter thus leading to apoptosis at different cell lines. However, there is still lack of in-depth morphological and immunophenotypical proof of the hypothesized restoration of differentiation after treatment with VPA. Aim. We aimed to characterize the differentiation effect of VPA on AML1/ETO positive leukemic cells and primary patients' blasts and to determine the expression pattern of selected AML1 target and candidate genes. *Methods.* Kasumi-1 (M2 AML AML1/ETO positive) cell line, Kasumi-6 (M2 AML AML1/ETO negative) and MV4-11 (M5 AML MLL/AF4 positive) cells were treated with VPA (0,5mM and 1,0mM concentrations) and 12-0-tetra-decanoylphorbol-13-acetate (TPA; 1,62nM concentration) and examined by flow cytometry and qRT-PCR. Z-VAD-FMK (caspase inhibitor; 20 and 100uM) was used to distinguish non-specific changes of immunophenotype during apoptosis. Two AML1/ETO positive patients' bone marrow diagnostic samples and two AML1/ETO negative samples were treated with VPA and TPA to confirm *in vitro* findings. *Results.* Valproic acid induced apoptosis in AML1/ETO positive and MLL/AF4 positive cells in dose dependent manner and G1/G0 arrest in AML1-ETO negative cells. But changes of immunophenotype proving the differentiation were observed purely in AML1/ETO positive cells. Particularly VPA treatment led to decreased expression of early myeloid progenitor antigens (CD33/34/117) and increased expression of antigens typical for differentiated myeloid cells (CD11a/11b). However, differentiated cells exhibited positivity of AnnexinV (early marker of apoptosis); hence the relationship between cell death and differentiation had to be evaluated. Despite the fact that Z-VAD-FMK reduced apoptosis by 80-85%, differentiation was detected in the same extent as previously with no AnnexinV positivity. Conversely changes in immunophenotype were not detected in either of control cells. TPA, which was used to exclude incapability of cells to differentiate, induced monocytic differentiation in both, AML1/ETO positive and negative cells. As quantified by qRT-PCR, VPA treatment increased gene expression in AML1/ETO-positive cells. Concretely VPA increased expression of PU.1($P<0.001$), C/EBPalpha($P<0.001$), BPI($P<0.001$) and IGFBP7($P<0.001$). No significant changes were detected in AML1/ETO negative cells. Specific effect of VPA was confirmed in patients'samples from the time of diagnosis. All samples exhibited changes of immunophenotype after TPA treatment while VPA treatment induced similar changes only in AML1/ETO positive samples. *Conclusions.* Taken together, we provide a valid evidence of differentiation specific for AML1/ETO positive cells accompanied by the increase of the repressed gene expression. Our findings of differentiation immediately followed by apoptosis bring a new insight to the biological effect of an old drug. In view of the ever more widespread use of HDACi in treatment of haematological malignancies, it is crucial to identify subgroups of patients who will benefit most from such treatment. Our data suggests that AML1/ETO positive AML patients are just such subgroup.

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GENE EXPRESSION PROFILE OF IMMUNE CITOKYNES IN PATIENTS WITH REUMATHOID ARTHRITIS UNDERGOING TREATMENT WITH IMMUNOADSORTIVE GRANULOCYTE/MACROPHAGE APHAERESIS

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Introduction. Rheumatoid arthritis (RA) is a systemic disease, mainly resulting in joint inflammation, but also increasing the risk for cardiovascular diseases. Although it is of unknown origin, immune system alterations could play an important role in its pathogenesis, probably due to a misbalance between Th1 and Th2 responses. Treatments options are focused into modulate the immune response with the use of biological agents. Recently, immunoadsorptive aphaeresis for granulocyte/macrophage has been applied for the treatment of these patients on the basis that it could induce a significant immunomodulation. However, clinical evidence and biological data providing information about this strategy is scarce. *Aims.* The aims of this study were: 1. To identify a specific expression profile of cytokines that regulate immune responses in patients with RA; 2. To study changes in this expression profile during and after treatment with immuoadsortive aphaeresis. Patients and *Methods.* Between January-08 and July-08, 16 consecutive RA patients who were refractory to standard treatment and who received treatment with immunoadsortive aphaeresis were included in the study. Additionally, x healthy controls were also included to compare mRNA cytokine expression between AR patients and controls. All patients underwent five aphaeresis sessions on a two sessions per week schedule. Samples for mRNA expression were collected in all cases before treatment start, after third session and at the end of treatment. mRNA expression of IL1, IL4, IL6, IL23, IL12, IL17, IFN-γ, TNFα, TGF β and FOXP3 were analyzed by means of TaqMan® gene expression assays using GUS and GAPDH as gene controls. *Results.* There were 14 females and 2 males. The median age for patients was 57 (range 31-74) and 21 (range 23-56) for controls. At the basal time, patients with RA showed higher levels of *vs.*G ($P<0.001$), alkaline fosfatase ($P=0.010$) and PCR ($P=0.001$) than controls. Similarly, significant differences were found in the expression of several cytokines with a key role in the immune response. Patients with AR showed a higher expression of IFNγ ($P<0.001$) and IL-1 ($P=0.07$) together with a lower expression levels for IL-4 ($P=0.006$) and TGF-β ($P=0.006$) than controls. So that, the expression pattern suggests that patients with RA have a misbalance between Th1 and Th2 immune responses, with an increase on Th1 activity. This specific expression profile did not show any significant change during or after treatment with immunoadsortive aphaeresis. *Conclusion.* Our results suggest that patients with RA could be characterized by a specific expression profile for cytokines that regulates immune responses. Th1 responses could be increased with respect to Th2 responses, which is compatible with the clinical manifestations of RA. Treatment with immunoadsortive aphaeresis for granulocyte/macrophage compartment did not induce changes in this expression profile. Taken into account these findings together with the limited clinical data, further clinical trials are required before including this treatment strategy in the algorithm of RA patient's management.

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AURORA-A KINASE INHIBITION ENHANCES THE CYTOSINE ARABINOSIDE-INDUCED CELL DEATH IN LEUKEMIA CELLS THROUGH APOPTOSIS AND MITOTIC CATASTROPHE

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Background. Aurora-A (Aur-A) is a centrosome-associated serine/threonine kinase that is overexpressed in various cancers and potentially correlated with chemoresistance, and Aurora kinase inhibition sensitized anti-tumor effects induced by chemotherapy. *Methods.* We evaluated the effect of Aur-A inhibition on the cytosine arabinoside (Ara-C)-induced cell death in the Ara-C-sensitive (Molt-3, HL60, KG1, NB4) and Ara-C-resistant (U937, K562) leukemia cell lines, respectively. *Results.* In the Ara-C-sensitive cell lines, silencing of Aur-A by small interfering RNA transfection (Aur-A siRNA) led to a significant increase in the Ara-C-induced cell death rate through induction of mitochondria-mediated, caspase-dependent apoptosis. In contrast, combined treatment of the Ara-C-resistant leukemia cells with Aur-A siRNA and Ara-C (Aur-A siRNA/Ara-C) remarkably enhanced the cell death rate via non-caspase-dependent mitotic catastrophe, which was accompanied by G2/M arrest, increase in the cells with >4N DNA content and multinucleation. Pretreatment of Ara-C-sensitive leukemia cells with a specific p38 MAPK inhibitor SB203580 abrogated the Aur-A siRNA/Ara-C-induced apoptosis, indicating the role of p38 MAPK pathway in the enhancement of apoptosis in these cells. *Summary/Conclusions.* Taken

together, Aur-A inhibition was an effective treatment for both the Ara-C-sensitive and resistant leukemia cells by increasing apoptosis and mitotic catastrophe, respectively. Further studies are necessary to evaluate whether Aur-A inhibition could be a useful therapeutic strategy for chemoresistant acute leukemia disorders.

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TARGETED INTEGRATION OF TRANSGENE INTO THE AAVS1 LOCUS ON CHROMOSOME 19 USING AAV INTEGRATION MACHINERY IN HUMAN MESENCHYMAL STEM CELLS

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Background. Mesenchymal stem cells (MSCs) are easily cultured from bone marrow, rapidly expanded *in vitro* and readily transduced with a variety of viral vectors, and have thus been used in early gene therapy experiments. MSCs have an ability to differentiate into various cell lineages and are attractive tools for regenerative medicine. Retroviral vectors are commonly utilized to introduce foreign genes into these cells. Although the transduced cells can express transgene for a long time, cells transduced with randomly integrating retroviral vectors may transform due to insertional mutagenesis. To date, the adeno-associated virus (AAV) has been identified that integrates its genome into a particular location in human chromosome 19 (19q13.4), known as AAVS1, through the activity of specific replicase/integrase protein, Rep, which can bind both the AAVS1 and the viral inverted terminal repeat (ITR) and mediate site-specific integration. *Aims and methods.* The AAVS1 site is a presumed hazard-free genomic location identified as the specific site. In the present study, we tested AAVS1-targeted insertion of a reporter gene in KM-102 cells (a stromal cell line established from human bone marrow cells) by transfection with a Rep plasmid and a bicistronic transgene plasmid containing a GFP gene under the control of CMV promoter and a blasticidin S resistance gene (bsr) under the transcriptional control of internal ribosomal entry site (IRES) flanked by the ITRs. *Results.* Southern blot analysis of blasticidin S resistant clones revealed that about 10% of the clones showed site-specific integration of the GFP gene into the AAVS1 site. We were also able to amplify the junction sequence between the GFP plasmid and the AAVS1 site. The AAVS1 locus overlaps with the first exon and intron of myosin binding subunit 85 (MBS85), which is involved in the assembly of actin filaments. These clones could grow well like the wild-type KM-102 cells but showed a decreased level of MBS85 mRNA. Summary/*Conclusions.* The results indicated that although the insertion of the transgene at AAVS1 affected MBS 85 expression, it did not appear to cause a phenotypic change of KM-102 cells. The use of the AAV integration machinery to insert a transgene into the AAVS1 locus will contribute to safe genetic manipulation of MSCs. This technology may also be applied to regenerative medicine using ES cells or iPS cells in the future.

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5-AZACYTIDINE AND 5-AZA-2´DEOXYCYTIDINE (DECITABINE) EXERT PROFOUND PRO-APOPTOTIC EFFECTS IN NEOPLASTIC HUMAN MAST CELLS

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Aggressive systemic mastocytosis (ASM) and mast cell leukemia (MCL) are advanced myelogenous neoplasms with a poor prognosis. In these patients, neoplastic mast cells (MC) are resistant against most conventional drugs. Demethylating agents reportedly exert beneficial effects in some advanced myelogenous neoplasms, including myelodysplastic syndromes. We examined the effects of two demethylating agents, 5-azacytidine and 5-aza-2'deoxycytidine (decitabine) on growth and survival (apoptosis) of neoplastic MC and the human MC line HMC-1. Two HMC-1 subclones were used, HMC-1.1 lacking KIT D816V and HMC-1.2 exhibiting KIT D816V. Both demethylating agents were found to induce apoptosis in HMC-1.1 cells and HMC-1.2 cells in a dose-dependent manner (ED50: 10-20 μM). In normal cultured MC and normal bone marrow cells, no substantial effects of 5-azacytidine or decitabine on growth or viability were observed. Drug-induced apoptosis in HMC-1 cells was accompanied by cleavage and activation of Caspase 8 and Caspase 3 as well as an increased expression of proapoptotic FAS/CD95, and decreased expression of KIT/CD117. Unexpectedly, as assessed by 3H-thymidine uptake, only decitabine was found to inhibit the proliferation of HMC-1 cells at pharmacologic drug-concentrations (IC_{50}: 1-5 μM), whereas no effect was seen with 5-azacytidine (>20 μM). Moreover, only decitabine but not 5-azacytidine, was found to induce a G2/M arrest in neoplastic MC. We were also able to confirm growth-inhibitory effects of decitabine on neoplastic cells in a patient with KIT D816V+ SM associated with CMML. Together, our data show that 5-azacytidine and decitabine exert differential effects on growth and survival (apoptosis) in neoplastic MC. Whether epigenetic drugs also produce anti-neoplastic effects *in vivo* in patients with ASM and MCL remains to be determined in clinical trials.

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NO SYNERGISTIC EFFECTS OF LENALIDOMIDE COMBINED WITH DASATINIB REGARDING ADCC INDEPENDENT NK CELL EFFECTOR FUNCTIONS AGAINST MM CELLS IN VITRO

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Background. Lenalidomide, an IMiD® (immunomodulatory drug) is well established for the treatment of relapsed/refractory multiple myeloma (MM) patients. Besides its direct anti-tumor effects it has been shown to modulate natural killer (NK) cell effector functions, especially those associated with antibody dependent cellular cytotoxicity (ADCC). The multi-kinase inhibitor dasatinib inhibits oncogenic SRC family kinases, which are associated with IL-6 induced proliferation in MM. Under certain circumstances dasatinib has been described to enhance NK cell effector functions. Currently, a clinical trial applying the combination of lenalidomide, dasatinib and dexamethasone for the treatment of MM (clinicaltrials.gov Identifier NCT00560391) is ongoing. *Aims.* We examined the potential for synergistic effects of the combination of lenalidomide and dasatinib on MM cell lines, primary MM cells and key effector functions of NK cells, because NK cells are the first line of defense against MM. *Methods.* Peripheral blood mononuclear cells (PBMCs) or expanded polyclonal NK cells *ex vivo* cocultured with irradiated RPMI 8866 feeder cells (both from healthy human blood donors after written informed consent was obtained) were analyzed *in vitro*. Furthermore, direct effects of the drug combination on primary MM cells and on the MM cell lines U266 and OPM-2 were evaluated. Functional outcomes assessed in NK cells included apoptosis/necrosis induction, cytotoxicity, degranulation marker CD107a/b expression and TNFα/IFNγ production. Proliferation behavior and/or apoptosis induction were assessed in MM cell lines and primary MM cells. *Results.* Dasatinib [50nM] alone and in combination with lenalidomide [50μM] significantly increased the apoptosis/necrosis rate on day 3 and 4 in PBMCs (n=4). However, it decreased the apoptosis/necrosis rate in expanded NK cells (n=5). Cytotoxicity, CD107a/b expression and cytokine production were unaffected by 24h pre-treatment with lenalidomide [1μM - 50 μM] (PBMCs: n=3-5; expanded NK cells: n=3, only 50μM tested). 24h pre-treatment with dasatinib with or without lenalidomide significantly reduced the cytotoxicity against MM cell lines in NK cells from PBMCs (OPM-2 n=3, U266 n=5), but not in expanded NK cells (n=3 for both target cell lines). However, it significantly increased the CD107a/b expression in expanded NK cells, when stimulated with MM cell lines (OPM-2 n=3, U266 n=3). In contrast, when dasatinib with or without lenalidomide was only present in the assay, cytotoxicity was significantly inhibited (expanded NK cells, n=3 independent experiments). Cell death induction in primary MM cells in coculture with stromal cells was heterogeneous, with 4/12 samples showing an enhancement by the combination of dasatinib [1 μM] and lenalidomide [20 μM], 4/12 samples showing no improvement over the dasatinib activity alone, and the remaining samples unaffected by either the single drugs or their combination. The combination of lenalidomide [20 μM] with dasatinib [2 μM] was more effective than the single drugs alone in OPM-2 cells with or without coculture with stromal cells, but had little effect on U266 cells. *Summary/Conclusions.* According to our results the combination of dasatinib and lenalidomide does not increase the direct, ADCC independent NK-cell activity *vs.* MM *in vitro* and seems therefore not to be a promising approach for clinical immunotherapeutic application, however further evaluations are ongoing.

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CYTOTOXIC EFFECTS OF BENDAMUSTINE IN COMBINATION WITH ANTILEUKEMIA / LYMPHOMA AGENTS AGAINST HUMAN MALIGNANT LYMPHOID CELL LINES

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Background. Bendamustine, unique cytotoxic agent that combines alkylating and antimetabolite activities, has shown significant activity against lymphoid malignancy. Although the recent advances in the preclinical and clinical use of bendamustine has been rapid, no conclusions can be drawn from the available data regarding its mechanisms of action, the optimal dose, and schedule and the optimal drugs to combine with it. *Purpose.* To investigate the optimal drugs with bendamustine in combinations, we studied the drug interactions of bendamustine with other antileukemia/lymphoma agents in human malignant lymphoid cell lines *in vitro*. *Methods.* Human B-cell lymphoma HBL-2 cells, B-cell leukemia BALL-1 cells, and myeloma U-266 cells were used for the study. The cells were simultaneously exposed to bendamustine and the other agents for 4-7 days. Cell growth inhibition was determined using the MTT reduction assay. The cytotoxic interactions at the IC80 level were evaluated by the isobologram method (Steel and Peckham). *Results.* The IC80 levels of bendamustine were 31 μM, 23 μM, and 10 μM, respectively, in these cell lines. Bendamustine produced synergistic effects with cytarabine, gemcitabine, and 4-hydroperoxy cyclophosphamide; additive effects with doxorubicin, etoposide, F-ara-A, mitoxantrone, and vincristine; and antagonistic effects with methotrexate in all three-cell lines. Cell cycle analysis using HBL-2 cells revealed that S-phase arrest was markedly induced when bendamustine was combined with cytarabine or 4-hydroperoxy cyclophosphamide. *Conclusions.* Our findings suggest that bendamustine has favorable antitumor effects when it is administrered simultaneously with most of studied. Among them, cytarabine, gemcitabine, and 4-hydroperoxy cyclophosphamide were more cytotoxic than expected when combined with bendamustine, and so these combinations are worthy of clinical investigation, whereas methotrexate may be inappropriate for simultaneous administration with bendamustine. These data provide useful information for the establishment of clinical protocols involving bendamustine.

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MICROVASCULAR BLOOD FLOW ALTERATIONS IS REVERSED BY LEUKAPHERESIS IN ACUTE LEUKEMIA: A CASE STUDY

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Background. Acute leukemia is primarily a bone marrow disorder that can be complicated by leukocytosis. Extreme leukocytosis can cause leukostasis due to sludging of circulating leukemic blasts into the microvasculature. Recently, this has been visualised by direct observation by SDF imaging.[1] Leukapheresis is used to correct hyperleukocytosis and leukostasis in the expectation of preventing microcirculatory obstruction. *Aims.* We hypothesized that this reversal of microcirculatory alterations could be observed by direct sublingual observation of the microcirculation by SDF imaging. *Methods.* In a 55 year old patient, recently diagnosed with acute myeloid leukemia (AML), was admitted to the intensive care unit with respiratory failure. Sublingual microcirculatory imaging was performed by using side stream dark field (SDF) imaging.[2] Sublingual capillary blood flow was estimated by off-line computer analysis using the semi-quantitative microvascular flow index (MFI).[3] *Results.* SDF-imaging showed a discontinuous microvascular blood flow pattern in all vessels before leukapheresis most probably caused by leukostasis (WBC count before leukapheresis 109×10^9/L; WBC count after leukapheresis 41×10^9/L). Only red blood cells can be observed by the SDF technique; the large spaces between the red blood cells suggest the occurrence of leukocytes between them. After leukapheresis the microvascular blood flow was normalized as reflected by increased MFI in all vessels. The improvement of blood flow was most obvious in the small vessels: MFI (small vessels before leukapheresis)= 0; MFI (small vessels after leukapheresis)=3. *Conclusion.* We conclude that leukapheresis is able to revert microcirculatory alterations in acute leukemia and that such effects can be directly observed by SDF imaging at the bed side.

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USE OF PROPRANOLOL IS EFFECTIVE AND SAFE IN TREATING INFANTILE HAEMANGIOMAS: REPORT OF 5 CASES

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Infantile haemangiomas (IH) are the most common vascular tumours of childhood. Usually their course is self - limiting. Albeit that, a significant minority requires treatment due to dramatic cosmetic impact or functional impairment. The efficacy of propranolol for the treatment of IH was recently serendipitously discovered. Since then, there has been a great interest in this off-label use of propranolol. We report our experience with this novel treatment in 5 children with infantile haemangiomas. The patients' age at treatment initiation was 2 months to 12 years. The haemangiomas were situated at the right forearm in 2 cases and at the right labium major, at the right cheek, uvula and right upper eyelid and at the tip of the nose in the remaining 3. Parents provided informed consent in all cases. Thorough cardiologic (clinical examination, electrocardiogram and echocardiography) and respiratory evaluation were carried out prior to initiation of treatment. Additionally, all patients had full blood count, biochemistry profile, urine dipstick for glucose, abdominal ultrasonography and ultrasonography of the IH treated. Propranolol was given every 8 hours, with an initial dose of 0.16 mg/kg/dose. The second day the dose was doubled and the third day the full dose of 0.66 mg/kg/dose was reached (total dose of 2 mg/kg/day). Parents were encouraged to feed their children frequently and anticipatory guidance was provided regarding symptoms and signs of hypoglycaemia, bradycardia and hypotension. Patients remained in hospital for 48 hours under close cardiorespiratory monitoring; fingerstick blood glucose was checked every three hours. In all cases oral propranolol led to rapid discoloration of the haemangioma, ulcer healing when present and size decrease of the lesion. No side effects were reported in any of the cases treated. Propranolol appears to be both effective and safe in the treatment of infantile haemangiomas. Large, prospective, well designed studies still need to verify these parameters as well as the best treatment regimen.

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EFFICACY OF RITUXIMAB FOR TREATMENT OF AUTOIMMUNE CYTOPENIAS ASSOCIATED WITH LYMPHOPROLIFERATIVE DISORDERS: THE MANITOBA EXPERIENCE

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Background. Autoimmune cytopenias (AIC) are a known complication of lymphoproliferative disorders (LPD). Treatment of AIC remains a therapeutic challenge. In recent years, the anti-CD20 monoclonal antibody rituximab has been used for the treatment of AIC refractory to or dependent on steroids or other immunosuppressants. Here we report our results of rituximab treatment for patients suffering from LPD associated AIC. *Aim.* To evaluate the efficacy of rituximab for the treatment of AIC in patients with an underlying LPD. *Methods.* All patients treated with rituximab for AIC associated with LPD in Manitoba between January 1, 2003 and December 31, 2007 were identified using the CancerCare Manitoba Pharmacy database and the Aria Information system (Varis Med Oncology Software). The charts of all these patients were reviewed retrospectively to assess response to therapy. *Results.* Rituximab was administered to 16 patients with LPD who developed AIC refractory to or dependent on steroids or other immunosuppressants. Details of the patients and outcomes are given in the table below. Of the 12 patients with autoimmune hemolytic anemia (AIHA), 10 had CLL, and 6 were Coomb's positive. All patients with immune thrombocytopenic purpura (ITP) had CLL. Rituximab was administered at a median time of 3, 13 and 4 months from the diagnosis of AIHA, ITP and AIHA + autoimmune neutropenia (AIN) respectively. A median of 4

cycles of rituximab were administered per course. All patients concomitantly received prednisone. Responses: (a) AIHA - 11 patients received a total of 15 courses of rituximab with a complete response (CR) in 80% at a median time of 2 months. Five patients achieving CR with the first course of rituximab relapsed at a median time of 17 months. Four of the five patients who relapsed received a second course of rituximab, and all four achieved a complete response. Of the 11 patients treated with rituximab, five died at a median time of 8 months post rituximab. Two of these patients died within 1 month of rituximab completion. Both these patients had advanced CLL. At last follow-up, two patients remained in remission at 3.5 and 18 months. (b) ITP - 4 patients received a total of 5 courses of rituximab with a CR in 60% and a partial response (PR) in 40% at a median time of 1 month. One patient who achieved CR with the first course of rituximab relapsed at 18 months and achieved CR with a second course of rituximab. Of the 4 patients treated with rituximab, 1 patient died at 6 months post rituximab completion. At last follow-up, 2 patients remain in remission at 25 and 26 months. (c) One patient treated for AIHA and AIN achieved CR at 2 months without relapse. This patient died at 30 months post rituximab. *Conclusion.* Rituximab therapy, regardless of regimen, appears to be safe and effective for the treatment of LPD associated AIC not responsive to conventional therapies. Patients who relapse following rituximab successfully respond to a second course of rituximab.

Table. Patient Characteristics and Response to Rituximab.

		AIHA	ITP	AIHA + AIN
Patients	Numbers	11	4	1
	Median age ritux started	70 years (range 56 -77)	69 years (range 61 - 84)	62 years
	Median lab values start of treatment*	Hgb 90g/L (range 61 - 132*)	Plts 97x10^9/L (range 18 - 147*)	Hgb 140g/L* ANC 1.9x10^9/L*
Number of courses per regimen	Total	15	5	1
	RCD	9	3	1
	R-CVP	3	–	–
	Ritux+/-steroid	3	2	–
Number of courses	One	7	3	1
	Two	4	1	–
Responses	Numbers (%)	CR: 12/15 (80%)	CR: 3/5 (60%) PR: 2/5 (40%)	CR: 1/1(100%)

RCD = Rituximab, Cyclophosphamide, Dexamethasone
R-CVP = Rituximab, Cyclophosphamide, Vincristine, Prednisone
Hgb = hemoglobin, Plts = platelets, ANC = absolute neutrophil count; Ritux = Rituximab
*high values due to ongoing steroid and/ or other immunosuppressants

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INVESTIGATION OF CYTOTOXIC EFFECTS OF AU(III), PT(II) COMPLEXES OF 5-CHLORO-1,10-PHENANTHROLINE ON HL-60 (ACUTE PROMYELOCYTIC LEUKEMIA CELLS) CELL LINE

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Backround. Au(III) complexes, the last isostructural and isoelectronic with platinum(II) complexes, are potentially attractive as anticancer agents. This, together with the recent finding that certain transitional metal complexes like Au(III) and Pt(II) complexes have been found to be potentially useful in cancer chemotherapy, created a renewed interest in the study of the interactions of metal ions with respect to the site of binding and the structure and stability of the complexes. *Aim.* The purpose of this study was to investigate the cytotoxic effect of Au(III) and Pt(II) metal complexes on human leukemic cell lines *Methods.* In this study Au(III) and Pt(II) metal complexes of 5-chloro-1,10-phenanthroline (5-Cl-phen) were synthesized and elucidated of their structure was performed by IR, 1H-NMR and MASS spectroscopic data and elemental analyses results. Then, we studied the antiproliferative effects of Au(III) and Pt(II) metal complexes of 5-chloro-1,10-phenanthroline, and cisplatin on HL-60 cell line. HL-60 was maintained in the RPMI1640 with % 10 heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 Iu/mL penicillin and 100 µg/mL streptomycin, at 37 °C in a humidified incubator containing %5 CO2. The antiproliferative effects of these compounds were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) cell viability assay by following the 24 and 48 hours incubation of leukemic cells in 5,10,25,50,100 ul concentration of Au(III) and Pt(II) complexes and cisplatin. All experiments were repeated three times. Cytotoxic effect depending on the concentration and time was viewed also by means of light microscope. *Results.* We determined that Au(III) and Pt(II) complexes of 5-chloro-1,10-phenanthroline, and cisplatin had shown significant the time- and concentration-dependent antiproliferative effects on the HL-60 cell line. When HL-60 cells exposered to 50 and 100 µL [Au(5-Cl-phen)Cl2]Cl for 24 h, the cell viabilites decreased to 30% and 43% respectively compared to control. After 48 h 50-100 µL [Au(5-Cl-phen)Cl2]Cl concentrations significantly decreased the cell viabilty ($P<0.001$). The concentrations of 50, and 100 µL [Au(5-Cl-phen)Cl2]Cl for 48 h, the cell viability percenties were determined to 44%, 55% respectively. HL-60 cells exposered to 50 and 100 µL [Pt(5-Cl-phen)Cl2]Cl and cisplatin for 24 h, the cell viabilites decreased to 24% and 42%; 16% and 26%. The concentrations of 50, and 100 µL [Pt(5-Cl-phen)Cl2]Cl and cisplatin for 48 h, the cell viability percenties were determined to 31%, 44% and 16%, 39% respectively Especially, [Au(5-Cl-phen)Cl2]Cl observed an inhibition of cancer cell proliferation higher than [Pt(5-Cl-phen)Cl2]. *Summary/Conclusions.* In conclusion, the present study demonstrates a powerful *in vitro* antitumor action of Au(III) complexes of 5-chloro-1,10-phenanthroline ([Au(5-Cl-phen)Cl2]Cl) on HL-60 cell lines.

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MITOCHONDRIA AS A MOLECULAR TARGET IN HEMATOLOGICAL NEOPLASIAS

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Current cancer therapies seek to induce cell death, but only a limited success has been accomplished mainly due to the lack of specificity for cancer cells. Therefore, it is important to investigate treatment strategies for selective tumor cell killing in hematological malignancies.

The primary function of mitochondria is oxidative phosphorylation which supplies the majority of cellular ATP required to sustain normal and tumor cellular functions. The selective inhibition of this process may therefore provide a novel and effective strategy for cancer treatment. On the other hand, considering that mitochondria are the main site for Reactive Oxygen Species (ROS) production, it has been reported that it may have a relevant role in inducing cell death. In fact, it is widely known that mitochondria is involved in apoptotic cell death and that neoplastic cells have an higher mitochondrial potential than normal cells, which may be explored in the development of new approaches to treat cancer. Lipophilic cations possessing a delocalized positive charge (delocalized lipophilic cations or DLCs) penetrate the hydrophobic barriers of the plasma and mitochondrial membranes and accumulate in mitochondria in response to the negative inside transmembrane potentials. The higher plasma and/or mitochondrial membrane potentials of neoplastic *vs.* normal cells, in general, account for greater uptake of these compounds in neoplastic cells and may be a way to selectively target these cells since DLCs exhibit mitochondrial toxicity at high concentrations. Dequalinium (DQA) is a DLC that interferes with mitochondria and so our goal is to evaluate the therapeutic potential of DQA in haematological malignancies, namely in B-cell Chronic Lymphocytic Leukaemia (B-CLL), Acute Promyelocytic Leukaemia (APL) and Myelodysplastic Syndrome (MDS). For this purpose we used three cell lines, EHEB (B-CLL), HL-60 (APL) and F36P (MDS), to evaluate the effect of different concentrations of DQA either by single dose administration, by daily dose administration and by association with conventional anticarcinogenic agents.Cell viability and death was determined by the resazurin assay, optical microscopy and by flow cytometry. The latter was also used to evaluate the mitochondrial membrane potential, the levels of ROS (H_2O_2; $O_2^{\cdot-}$) and the antioxidant defense, Reduced Glutathione (GSH), using fluorescent probes. We found that DQA induced a decrease in cell viability inducing cell death by late apoptosis/necrosis in a time, dose and cell type dependent manner, with an IC_{50} of 2.5, 5 and 7.5 µM at 48h of exposure, respectively to HL-60, F36P and EHEB. These effects may be mediated by oxidative stress as we have observed an increase in ROS production and a decrease in GSH levels and in mitochondrial membrane potential. We also observed that if DQA is administered on a daily basis a much lower concentration is required to induce the same effect. On the other hand, the association of DQA with the conventional drug induces a synergistic effect, because lower concentration of both drugs is required to obtain the same effect. In summary, our results suggest that DQA may be used as new therapeutic approach in hematological neoplasias both in monotherapy and in association with conventional therapy.

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LANGERHANS CELL HISTIOCYTOSIS IN CHILDREN - TREATMENT OF PATIENTS WITH REACTIVATION OF DISEASE

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Background. Langerhans cell histiocytosis (LCH) is a rare disease. Despite advances in the study of LCH, the etiology and pathogenesis are poorly understood and the treatment is still not standardized. It is regarded as a clonally accumulation and proliferation of abnormal bone marrow derived Langerhans cells. *Aims.* To present the experience of University Children's Hospital in Skopje regarding to clinical features, diagnosis, treatment and follow-up of LCH in children with a reactivation of disease. *Methods and results.* During the period from January 1977 to January 2010, 55 cases with proven histology LCH (31 males and 24 females) were analyzed and treated at the Department of Hematology/Oncology. The age at diagnosis ranged from 3 to 192 months. Seven of fifty five children presented skin involvement, 8/55 presented single bone lesion, 7/55 had multiple bone lesions and 33/55 presented multisystem diseases (13 of them had organ dysfunction). Because of a very long period of analysis there were different treatment procedures: combination of Vinblastine and Prednisone, LCH-I, LCH-II and from 2003 we started to use LCH-III Protocol. Six patients were treated according to LCH-III. One patient 4 months old with multisystem disease and organ dysfunction died during the Consolidation Treatment. Three patients finished the treatment with LCH-III and they are in remission, without disease 1 month, 18 months and 7 years respectively. Two children manifested reactivation of disease. The first one was 17 years boy with multiple reactivations of multifocal bone LCH, with no involvement of risk organs. He was treated with LCH-III Protocol for the primary disease and for the first reactivation; and with Low Risk 2nd Line Treatment Initial and Consolidation Therapy for the second reactivation of disease. He received also a treatment with bisphosphonates because of very severe pain and osteoporosis produced by treatment. One year after cessation of therapy he is in Complete Remission. The second case was 6 years old girl with LCH disease with multiple reactivations. The primary disease started at the age of 7 months with vulvovaginitis recidivist, involvement of the inguinal lymph nodes, liver, skin and bones, without involvement of the lungs. She was treated with LCH-III protocol. First reactivation of the disease was manifested 9 months after the end of LCH-III Protocol involving the bones, skin, and pituitary involvement with Diabetes insipidus. She started again with LCH-III Protocol. Eight months after the end of LCH-III Protocol the child manifested soft tissue lesions of the skull with extensions of the bones. She started with Low Risk 2nd Line Initial Treatment on February 2010, with a very prompt response to the treatment. *Conclusion.* Reactivation of LCH is defined by the appearance of new sites of active disease in the same or different organs of patient in whom the signs and symptoms of the previous episode have disappeared. There is not a single strategy of treatment, and different approaches could be effective in these cases. The schedule Low Risk 2nd Line Treatment Initial and Consolidation Therapy is expected to offer good results for patients with LCH reactivation.

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EVALUATION OF THE THERAPEUTIC POTENTIAL OF A RECOMBINANT TRAIL IN LEUKEMIA - A STUDY IN CELL LINES IN CULTURE

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Recent progress has broadened us to think that deficient apoptosis is a major cause in the development and progression of cancer, namely in leukemias, and it plays a significant role in the resistance to conventional therapeutic regimes. On this way, apoptosis may be a useful target for therapeutic intervention. The tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL/Apo-2L) is a member of the TNF superfamily that trigger and activate 4 receptors, TRAIL-R1 and -R2, also known as death receptors, DR4 and DR5, and antagonistic receptors, TRAIL-R3 and-R4, also known as decoy receptors, DcR1 and DcR2. Several studies demonstrated that TRAIL can induces cancer cell death with low cytotoxity in normal cells, but few have been done in Leukemias. The aim of this work is to analyse the therapeutic efficacy of a recombinant TRAIL (rhAPO2L/TRAIL) in haematological cancer, namely in acute and chronic leukemias. For this purpose we use several leukemia cell lines such as: Acute Myeloid Leukemia (HL-60), Chronic Myeloid Leukemia in blast crisis (K-562), Acute Lymphocytic Leukemia T (MOLT-3) at presention, Acute Lymphocytic Leukemia T cells in remission (MOLT-4) and Chronic Lymphocytic Leukemia B (EHEB). These cell lines were treated with different concentrations of rhAPO2L/TRAIL and the viability was measure using the trypan blue method. Cell death analysis was performed by flow cytometry (Annexin V/Propidium Iodide) and Optical Microscopy. TRAIL and TRAIL-Rs were evaluated by Flow cytometry using monoclonal antibodies. Our results show that rhTRAIL induced a decrease in cell viability inducing cell death, in a time, dose and cell type dependent manner. In fact, we observe an IC_{50} in HL-60 and EHEB cells treated during 48h, respectively, with 250 ng/mL and 750 ng/mL of rhTRAIL. On the other hand, the IC50 in MOLT-3 and MOLT-4 cells were obtained earlier, after 24h of rhTRAIL incubation, and at a lower concentration (~250 ng/mL). However, after this incubation period, in ALL-T cell lines we observed a reversion on the rhTRAIL cytotoxic effect. In K562 cells, rhTRAIL wasn't able to induce a significant decrease in cell viability in monotherapy. Beside that, when we compared the effect obtained in all cell lines, we observe that acute leukemic cells (HL-60, MOLT-3 and MOLT-4) were more sensible to rhTRAIL then chronic ones (K562 and EHEB), after 24 hours treatment. However, the cytotoxic effect is maintained only in the HL-60 and in EHEB cells. These results may be correlated with the differential TRAIL receptors expression, namely the presence of the anti-apoptotic TRAIL receptors, TRAIL-R3 and TRAIL-R4, in K562 cells. On the other hand, the higher percentage of pro-apoptotic TRAIL receptors, TRAIL-R1 and TRAIL-R2, may be related with the therapeutic efficacy of rhTRAIL in the other cell lines. In conclusion, our preliminary study suggests that rhTRAIL can be use as a new therapeutic aproach in the treatment of Leukemia, namely in ALL-T, B-LLC and APL, as single agent. However, the potential use of the drug in association with conventional therapy and the schedule of drug administration must be tested.

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MANUFACTURING OF BONE MARROW-DERIVED MESENCHYMAL STROMAL CELLS ACCORDING TO GMP REQUIREMENTS FOR CLINICAL TRIALS

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Background. Mesenchymal stromal cells (MSCs) are nonhematopoietic multipotent cells residing in bone marrow with the capacity to self-renew and to differentiate into multiple lineages. They display immunomodulatory, pro-angiogenic and reparative properties that were demonstrated in both animal and human models and has generated markedly increasing interest in a wide variety of biomedical disciplines. The International Society for Cellular Therapy (ISCT) proposed minimal criteria to define human MSCs: MSCs must be plastic-adherent, have a phenotype of $CD105^+$, $CD73^+$, $CD90^+$, $CD45^-$, $CD34^-$, $CD14^-$, $HLA\text{-}DR^-$, and have the capacity to undergo *in vitro* differentiation to osteoblasts, adipocytes, and chondrocytes. *Aims.* The objective of this work was to establish optimized protocol for manufacturing of GMP-graded MSCs that are meeting the ISCT criteria, are high-quality and safe and achieve number applicable in cellular therapy of Crohn's disease, limb ischemia and graft-versus-host disease. *Methods.* Techniques for MSCs isolation from the bone marrow, seeding density, expansion, cryopreservation, transport, quality control and potency testing had to be optimized for standard laboratory and subsequently for Clean rooms. To maintain phenotypic and genotypic stability of MSCs during multiple passages, optimization of culture conditions was one of the most crucial aims of MSCs GMP production. It was necessary to establish humanized serum free cell culture system without risk of ineligible immune reaction and transmission of zoonoses. *Results.* The optimal method for MSCs isolation was density gradient centrifugation of mononuclear cells on Ficoll Paque Premium 1.073 g/mL, and separation of MSCs by plastic adherence. The best expansion potential was achieved in medium DMEM supplemented with 5% platelet lysate. MSCs were harvested by trypsin and replated in density 3×10^5 cells per cm^2. Sequential passages might affect the quality and proliferation rate

of MSCs, therefore, no more than 4 passages are advisable. In clinically produced MSCs, attention must be paid to aseptic conditions and their validation. Controls of the process qualifying the methods at the time of process validation include potency tests of the MSC immunomodulatory effect in mixed lymphocyte reactions and tests of genetic stability even in higher passages. Controls of the production as contamination control and visual control of morphology and confluence are carried out during the cell expansion; cell number and viability are monitored at every cell harvest. Release criteria take into account the effectiveness and safety of the product: cell number according to indication (10-50×10^6 cells for local and 1-5×10^6 per kg for systemic administration), ≥80% viability and immunophenotype fulfilling ISCT criteria and excluding impurities ($CD3^-$, $CD19^-$, $CD80^-$). *Conclusions.* MSCs are resident in the bone marrow in small numbers of nucleated cells (0.001 0.01%), but can be readily expanded *in vitro* to provide sufficient number of GMP grade cells for local and systemic cellular therapy. The methods used in the manufacturing protocol will be validated and the clinical protocol will be approved by Czech State Institute for Drug Control.

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SENESCENCE INDUCTION THERAPY FOR ADULT T-CELL LEUKEMIA

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Background. It has been reported that retinoid (all-trans retinoic acid: ATRA or tamibaroten: Am-80) inhibits growth in HTLV-1-positive T-cell lines and fresh cells from patients with adult T-cell leukemia (ATL) *in vitro*. The retinoid therapy for ATL patients also has been evaluated in clinical status. In this study, we focused in role of cellular senescence in the treatment with retinoid. *Methods.* 1. Cellular senescence was observed by senescence-associated β-galacsidase (SA β-Gal) staining. 2. Gene expression of cyclin dependent kinase, Tax was performed by RT-PCR. 3. To determine whether or not telomere attrition was occurred, TRAP assay was performed. *Results.* 1. SA β-Gal positive cells were observed in spontaneous culture without retinoid (ATRA or Am-80) on HTLV-I (+) T-cell lines (HUT102, MT-2, MT-4, ED40515 and ATL-2) or primary cells from acute ATL patients, but not on HTLV-I (-) T cell lines (Jurkat and MOLT-4). Percent of SA β-Gal positive cells in ED40515 was lower than that of other HTLV-I (+) T-cell lines. By treatment with ATRA or Am-80, those number of SA β-Gal positive cells was increased significantly on both HTLV-I (+) T-cell lines and primary cells from acute ATL patients. However, there was no increase of SA β-Gal staining on HTLV-I (-) T-cell lines (MOLT-4 and Jurkat) even if treated with the retinoids. 2. Expression of p16INK4a was enhanced in all of HTLV-I (+) T-cell lines, but not in HTLV-I (-) T-cell lines. 4. In TRAP assay, no inhibition of telomere activity was observed in retinoid treated HTLV-I (+) T-cell lines, indicative of premature senescence. *Conclusion.* We found cellular senescence phenomenon in HTLV-I (+) T-cell lines and fresh primary cells from acute ATL patients. It has been reported that oncogene induced stress (OIS) induced cellular senescence. In this study, grade of cellular senescence on HUT102, MT-2, MT-4, and ATL-2 was greater in compared to on ED40515 that does not express Tax gene in mRNA level by nonsense mutation. By this fact, Tax gene may act as an oncogene. Furthermore, retinoid facilitated the cellular senescence. As mentioned above, chemotherapy using anti-neoplastic agents possibly decrease OIS and release cellular senescence results in rejuvenation of those cells and chemotherapy resistance.

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IAP INHIBITORS INDUCE APOPTOSIS IN CHILDHOOD ACUTE LEUKEMIA CELLS VIA NFκB-ACTIVATION AND TNFα-SECRETION AND OVERCOME BCL-2-MEDIATED RESISTANCE

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Despite enormous progress in the treatment of childhood acute leukemias, high risk acute lymphoblastic leukemias still do not respond well to current treatments. Because this failure is, in part, due to defects in apoptosis programs, new strategies are required that counter apoptosis resistance in order to improve the poor prognosis of high risk pediatric acute leukemia. Since "Inhibitor of Apoptosis"proteins (IAP) are expressed at high levels in acute leukemia and block apoptosis at a central point of the apoptotic machinery, they may present a suitable molecular target for therapeutic intervention. Here, we report that neutralizing IAP proteins by small molecule IAP inhibitors is a novel and effective approach to sensitize childhood acute leukemia cells for death receptor- or chemotherapy-induced apoptosis. IAP inhibitors at subtoxic concentrations, but not a structurally related control compound, synergize with TRAIL, agonistic anti-CD95 antibodies or various anti-leukemic drugs, to induce apoptosis in acute lymphoblastic leukemia cells. Further, IAP inhibitors act in concert with TRAIL, anti-CD95 antibodies or chemotherapeutic drugs to reduce clonogenic growth of ALL cells demonstrating that they also suppress long-term survival. Analysis of signaling pathways reveals that IAP inhibition triggers activation of NFκB and secretion of TNFα. IAP inhibitors increase death receptor- or chemotherapy-induced activation of caspases, loss of mitochondrial membrane potential and cytochrome c release in a caspase- and TNFα-dependent manner. Intriguingly, IAP inhibitors promote TRAIL-mediated caspase activation, mitochondrial perturbations and apoptosis regardless of high Bcl-2 expression by enhancing Bcl-2 cleavage and Bak conformational change. Thus, IAP inhibitors in combination with TRAIL even break Bcl-2-imposed resistance, a defect in the apoptotic pathway that is common in acute leukemia and associated with poor prognosis. In contrast to malignant cells, IAP inhibitors at equimolar concentrations alone or in combination with TRAIL are non-toxic to normal peripheral blood lymphocytes despite surface expression of the apoptosis-inducing TRAIL receptors, pointing to a therapeutic index. Of note, IAP inhibitors also kill primary leukemic blasts from children with ALL *ex vivo* and cooperate with TRAIL, anti-CD95 antibodies or chemotherapeutic drugs to induce apoptosis in primary leukemia cells. Most importantly, IAP inhibitors significantly reduce leukemic burden *in vivo* in a mouse model of pediatric ALL engrafted in NOD/SCID mice. Thus, IAP inhibitors present a promising novel approach for apoptosis-based therapy of childhood acute leukemia, which warrants further exploitation.

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G-CSF-INDUCED BONE MARROW-DERIVED STEM CELLS MOBILIZATION BEFORE SACROCOCCYGEAL PILONIDAL CYST SURGERY

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Stem cells, in particular the easily accessible bone marrow-derived stem cells (BMSC), may favour wound healing in patients requiring surgical excision for sacro-coccygeal pilonidal cyst (SPC). The present study was undertaken to evaluate feasibility, tolerability and safety of BMSC mobilization with granulocyte-colony stimulating factor (G-CSF) administration in patients undergoing surgery for complex SPC. *Patients and methods.* Eight patients with complex pilonidal disease were included in this prospective phase I-II study. All patients were treated with G-CSF (5 μg/kg b.i.d) for three consecutive days. Standard surgical excision of the pilonidal cyst was scheduled on day 2 of mobilization. Mobilization was assessed in terms of circulating $CD34^+$ cells (flow cytometry) and myeloid CFU-GM progenitors (*in vitro* colony growth assay). Patients were monitored for adverse events and clinical outcome. *Results.* $CD34^+$ cell mobilization occurred in all patients. Expansion of circulating CFU-GM progenitors along with a marked increase in white blood cells (WBC) was present, with a median peak value of 28,435 WBC/μL on day 3 of mobilization. G-CSF was well tolerated; no adverse events occurred. All patients received the planned surgical treatment without any complications. Overall, the clinical outcome was positive in terms of symptoms, recurrence and time required for wound healing. *Conclusions.* The association of G-CSF and surgery in patients with SPC is feasible, safe and well tolerated without pre- or post-operative complications. Morevoer these preliminary clinical results compare favourably with results observed in historical controls but need to be confirmed by randomized clinical trials assessing the efficacy of G-CSF administration in patients undergoing surgery for SPC.

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THERAPEUTIC EFFECT EVALUATION OF MONOMERIC AND POLYMERIC FLAVONOIDS, AND PHENOLIC ACIDS IN ACUTE PROMYELOCYTIC LEUKEMIA

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The consumption of bioactive compounds is recommended for the prevention of chronic diseases, including cancer and its preventive effect is evidenced in several epidemiological studies. However, the effect of bioactive compounds present in fruit and vegetables at the cellular and molecular levels remains poorly understood. Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia characterized by an accumulation of abnormal promyelocytes related with the t(15;17)(q22;q12), a chromosomal translocation that contributes to cell differentiation deregulation. Several studies have clearly demonstrated that many natural bioactive compounds can interfere with multiple cell signaling pathways including proliferation, survival, differentiation and even cell death by apoptosis. These effects might be mediated by oxidative damage, cell cycle alteration, hypoxia and cell adhesion loss. Through modern scientific approaches and innovative scientific tools is possible to demonstrate the promising pharmacological activity of polyphenolic fractions obtained from medicinal plants such as Cymbopogon citratus (Cc), a native herb from India. However, there aren't any studies in hematological neoplasias, namely in APL. The aim of this work is to evaluate the potential therapeutic effect of the three different polyphenolic fractions obtained from Cc leaves, containing flavonoids, proanthocyanidins (polymeric flavonoids), and caffeic and p-coumaric acid and their derivatives in acute promyelocytic leukemia. For this purpose, a Cc leaves infusion was submitted to fractionation, monitored by TLC and HPLC, to providing those three polyphenolic fractions referred above. The HL-60 cell line, obtained from an APL patient, was cultured in absence and presence of several concentrations of the three polyphenolic fractions and of the initial infusion, for different periods of time. Cell growth and viability were evaluated by the Trypan Blue assay and the efficacy of the drugs determined by the IC50. Cell death analysis was performed by morphological studies and by flow cytometry, using annexin-V/propidium iodide incorporation. The mechanisms involved in cellular cytotoxicity were analysed by flow cytometry through the expression of proteins involved in apoptotic pathways, namely Bcl-2, Bax, Fas, Fas ligand, Caspase 3 and P53. The mitochondria function and oxidative stress evaluation were analyzed by FC, through the determination of mitochondria membrane potential, ROS production and GSH levels, respectively using the JC1, DFCFH-HA and Mercury Orange fluorescent probes. Our preliminary results show that all drugs assayed induce a decrease in HL-60 cell viability in a dose, time and fraction composition dependent manner, with an IC50 values ranging from 50 to 250 ug/mL, after 48h of incubation. Caspase 3, Fas ligand and mitochondria may be involved in apoptotic cell death induced by these compounds since we observed a significant increase in the expression of these pro-apoptotic proteins and in mitochondrial membrane depolarization. In summary, flavonoids, proanthocyanidins, caffeic and p-coumaric acid and their derivatives can be active principles for drugs development that could be used as a therapeutic approach in acute promyelocytic leukemia.

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THE ROLE OF EPIGENETIC MODULATORS AS A THERAPEUTIC APPROACH IN LYMPHOID LEUKEMIAS - A COMPARATIVE STUDY

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The hypermethylation of the 'CpG islands' and histone deacetylation have been considered key epigenetic mechanisms in the silencing of genes, namely tumour suppressor genes, and may play a crucial role in several types of cancer, as in Acute and Chronic Lymphoid Leukemias. Unlike genetic changes, which are irreversible, epigenetic changes are reversible, allowing the expression or repression of a gene that might reverse malignant phenotype. Despite hypomethylating agents are already used in the treatment of some subtypes of Myelodysplastic Syndromes, and some studies are done in acute leukemias, the role of epigenetic modulators in the treatment of lymphoid leukemias (LL), particularly in Chronic Lymphocytic Leukemia (CLL) is still unclear. With this work we intend to evaluate the potential therapeutic effect of epigenetic modulators in lymphoid leukemias, namely in a CLL cell line in culture, as single agents and in combination and to compare this efficacy with those obtained in Acute Lymphoblastic Leukemia (ALL) cell lines. For this purpose, a CLL cell line obtained from a patient 69 years old, the EHEB cells, was maintained in culture in the absence (control) and presence of the demethylating drug, Decitabine (DEC), and/or with the histone deacetylase inhibitor, Trichostatin A (TSA), in several concentrations and during variable periods of time. The density and cell viability were assessed using the Alamar Blue assays, and cell death was analysed by flow cytometry, staining the cells with Annexin V. These results are then compared with those obtained previously in our laboratory in two ALL cells lines, the MOLT-3 and MOLT-4 cells, obtained from a patient at disease presentation and at relapse, respectively. Our results show that epigenetic modulators, TSA and DEC, induce a decrease in EHEB cells proliferation and viability in a dose and time dependent manner, inducing cell death preferentially by apoptosis. However TSA was more effective in monotherapy than DEC, reaching the IC_{50} at 250 nM after 72h of incubation, while with DEC the IC_{50} was not reached, besides the higher doses used. However, a decrease about 40% in cell proliferation was observed in EHEB cells treated with the higher DEC dose (100 μM). Furthermore, when we compared the efficacy of these drugs obtained in LLC cells with those obtained previously in our lab by AB Sarmento-Ribeiro *et al*. (2008) and Costa C *et al*. (2009) in ALL cells lines, we note that, besides TSA was also more effective in monotherapy than DEC, the cytotoxic effect is achieved later in EHEB cells, for the same drug concentration. Besides that, when TSA was administered to CLL cells 4 hours before than decitabine, it was observed a slightly increase in the cytotoxic drugs effect but at lower level than that observed in ALL cell lines. This study suggests that epigenetic modulation may become a new therapeutic approach in Chronic Lymphocytic Leukemia and Acute Lymphoblastic Leukemia, including relapse. However, the schedule of drugs administration and cell type may interfere with their therapeutic efficacy.

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LIPOSOMAL CYTARABINE AS NEUROMENINGEAL PROPHYLAXIS IN BRAIN MALT LYMPHOMA

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Background. Althougt a very rare entity, brain MALT lymphoma cases have been reported. *Aims.* to assess the safety, tolerability and efficacy of liposomal cytarabine as prophylaxis against lymphomatous meningeal infiltration. *Methods.* a 51 year old patient (pt), with Still's disease and bronchial asthma history, was admitted to our hospital in august 2007 by presenting partial seizures, with somatosensory discomfort in his lower right extremity (LRE) as starting symptom. Cranial MRI showed a mass in the falx cerebri, 3×2 cm, surrounded by a thin, hyperintense halo on the left side, which remained unchanged after three months. Differential diagnosis included chronic inflammatory pachymeningitis, Rosay-Dorfman disease and encapsulated abscess. It was decided to wait and see. After nine months the pt was readmitted presenting a partial seizure in his LRE. Cerebral MRI showed no changes in the dural lesion of the falx cerebri. Craniotomy was performed, with histopathologic diagnosis of NHL, primary extranodal marginal MALT with meningeal involvement, $CD20^{+}$, $CD5^{+}$, $CD43^{+}$, $bcl2^{++}$, Kappa and Lambda:$^{++}$. Postoperative imaging study in june 2008 showed hypersignal in right cerebral hemisphere, finding similar to that found previously in the contralateral side. After confirming that the extension study was negative, including cytology and flow cytometric immunophenotyping of cerebrospinal fluid, the pt received systemic chemotherapy: 5 well tolerated cycles of Rituximab (500 mg/m^2 day 1), Methotrexate (3,5 g/m^2 day 2) , Vincristine (1,4 mg/m^2 day 2) and Procarbazine (100 mg/m^2 days 1 to 7). In addition neuromeningeal prophylaxis with liposomal cytarabine (three 50 mg doses separated every four weeks) was

performed, associated with intrathecal hydrocortisone (10 mg) as prophylaxis of chemical arachnoiditis; no side effects were reported. *Results.* Reassessment MRI showed complete remission of the right mass, with significant reduction in postoperative collection on the left one. Afterwards holocraneal radiotherapy (45 Gy total dose) was administered. Currently the patient is asymptomatic and without evidence of lymphomatous disease. *Conclusions.* In our experience liposomal cytarabine may be safely used for prophylaxis of meningeal infiltration in patients with MALT lymphoma of the brain.

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CLINICAL RESULTS OF BENDAMUSTINE PLUS RITUXIMAB FOR INDOLENT NHL AND CLL.EXPERIENCE OF ONLY ONE INSTITUTION

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Introduction. Promising results have been reported in studies evaluating the combination of Bendamustine and Rituximab (B-R) in patients with relapsed/refractory indolent or mantle cell lymphomas. *Patients and methods.* we have analysed the role of the combination B-R in 8 patients since January 2009. They received Rituximab 375mg/m^2 (day 1) plus Bendamustine 90-100 mg/m^2 (days 1+2) every 28 days for a maximum of 6 cycles. The median patient age was 66 years (56-77 range). Most patients were in advanced stages (III-IV). Histologies were distributed: MALT NHL 12.5%, mantle cell NHL 37.5%, CLL/lymphocytic lymphoma 50%. Patients were heavily treated with a median of 3 prior regimens, including anthracycline containing chemotherapy (n=4) and purine analog chemotherapy (n=6). All patients had received previously rituximab. *Results.* Of the 8 patients, 4 are going in the treatment, they have not yet evaluated. A median number of 5 cycles was given (1-6 range). At the time of analysis February 2010, the median observation time was 6 months (1-13). Overall response rate for patients treated with B-R 100%. The CR rate was 75%. One patient progressed at 4 months. None patient died. Hematologic toxicities were observed for neutropenia grade 3+4. 15% cycles of bendamustine required patients admission in hospital. The B-R regimen was good tolerated by the patients, as evidenced by a lower rate of alopecia, number of infectious complications and estomatitis. We observed drug-associated erythematous skin reaction (rash) in one patient. There is not association between prior purine analog chemotherapy. *Conclusions.* the combination of Bendamustine +Rituximab have a excelent tolerability profile, and CR rate in heavily treated patients with relapsed/refractory indolent or mantle cell lymphomas. Further follow-up will determine whether the high RC/RCu rate corresponds to prolonged PFS. Additional updates on response will be available at the time of presentation.

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EFFICACY OF RITUXIMAB FOR TREATMENT OF AUTOIMMUNE CYTOPENIAS: THE MANITOBA EXPERIENCE

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Background. The treatment of autoimmune cytopenias (AIC) remains a therapeutic challenge. In recent years, the anti-CD20 monoclonal antibody rituximab has been used for the treatment of AIC refractory to or dependent on steroids or other immunosuppressants. Here we report our results of rituximab treatment for patients suffering from AIC in the absence of a lymphoproliferative disorder. *Aim.* To evaluate the efficacy of rituximab for the treatment of AIC in patients without an underlying lymphoproliferative disorder. *Methods.* All patients treated with rituximab for AIC in Manitoba between January 1, 2003 and December 31, 2007 were identified using the CancerCare Manitoba Pharmacy database and the Aria™ Information system (Varis Med Oncology Software). Patients with an underlying diagnosis of a lymphoproliferative disorder were excluded from analysis. The charts of all these patients were reviewed retrospectively to assess response to therapy. *Results.* Rituximab was administered to 5 patients with AIC refractory to or dependent on steroids or other immunosuppressants. Details of the patients and outcomes are given in the table below. Of the 3 patients with autoimmune hemolytic anemia (AIHA), all were Coomb's positive. One patient with AIHA received 4 courses of rituximab. All other patients received only one course of rituximab. All patients received 4 cycles of weekly rituximab per course except for one patient with Evans syndrome who received only 1 cycle of rituximab for immune thrombocytopenic purpura (ITP). Responses: (a) AIHA - 3 patients received a total of 6 courses of rituximab with a complete response (CR) in 67% and partial response (PR) in 33% at a median time of 2 months. One patient achieving CR with the first course of rituximab relapsed at 9 months. This patient received 3 subsequent courses of rituximab, subsequently achieving CR twice and PR once. The patient remains in remission at last follow-up 9 months post rituximab. Two of the three patients treated with rituximab died at a median time of 3 months post rituximab. Of these two, one patient died within 1 month of rituximab completion due to previously known hypotension attributed to autonomic neuropathy. (b) ITP - 2 patients received a total of 2 courses of rituximab with a complete response of 50% at a median time of 1 month. *Conclusion.* Rituximab therapy appears to be a safe and effective therapy for AIC not responsive to conventional therapies. Patients who relapse following rituximab, successfully respond to a second course of rituximab.

Table. Patient Characteristics and Response to Rituximab.

		AIHA	ITP
Patients	Numbers	3	2
	Median age when rituximab started	73 years (range 50 to 76)	26 years (ages 10 and 41)
	Median time from AIC diagnosis to rituximab initiation	16 months (range 9 to 19)	17 months (range 1 to 32)
	Median pertinent lab values at start of treatment	Hgb 88g/L (range 75 to 103)	Plts 8x10^9/L (range 3 to 12)
Number of courses	Rituximab+steroid	6	2
Responses	Numbers (%)	CR: 4/6 (67%) PR: 2/6 (33%)	CR: 1/2 (50%)
Relapses	Numbers	3	1
	Median time to relapse	8 months	30 months
Deaths	Numbers	2	0
	Median time to death	3 months	–

Hgb = hemoglobin, Plts = platelets, CR = complete response, PR = partial response

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EFFICACY OF INTRAPLEURAL (IP) RITUXIMAB (RMAB) IN PATIENTS (PT) WITH MALIGNANT PLEURAL EFFUSION (PE)

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Background. To date several agents have been used in performing pleurodesis in pleural lymphomatous infiltration, but there is little experience with the use of monoclonal antibodies. *Aims.* to evaluate the efficacy and safety of IP administration of anti-CD20 antibody. *Methods.* a 39 years old pt was diagnosed by gastric biopsy of non-Hodgkin lymphoma (NHL), diffuse large B cell with CD20$^+$ expression. Physical examination revealed multiple lymphadenopathies (submaxillary, cervical and supraclavicular), hypophonesis in both hemithorax and palpable splenomegaly. Cell blood count and blood biochemistry were normal. Lues, HIV, HBV, HCV, CMV, EBV, Toxoplasma and Rubella serologies all proved negative. A total body CT scan was performed and found submandibular, internal jugular, supraclavicular, right paratracheal, and retroperitoneal enlarged lymph nodes, bilateral PE and homogeneous splenomegaly. The pt began treatment with chemotherapy (ChT), R-CHOP-14 schedule. Because of massive PE we proceeded to endothoracic tube placement, presenting acute respiratory failure, which made it necessary tracheal intubation and ICU admission. Bilateral endothoracic tubes were placed, draining more than 1700 mL/day each. Once administered the second cycle of ChT left PE disappeared and left tube was removed; persistent right PE (draining 300 mL/day) prevented from right hemithorax extubation. After obtaining informed consent from the pt, pleurodesis with IP administration of Rmab was performed, as described: on the day 0, after premedication with chlorpheniramine maleate and corticoids, a dose of 50 mg of Rmab in 50 mL of saline (SF) was injected as a bolus in the right pleural cavity and then the tube was clamped for 6 hours (hs); on the day +1 a new dose of 150 mg of Rmab in 100 ml of SF, with subsequent clamping for another 6 hs. *Results.* treatment was well tolerated; after 72hs the right PE resolved

completely and the chest tube could be removed. *Conclusions.* IP administration of Rmab is safe and effective in controlling malignant PE in patients with NHL which express CD20+.

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RIBAVIRIN MADE EASY! DEVELOPMENT OF A GUIDANCE DOCUMENT AND EDUCATIONAL DVD FOR THE PREPARATION AND ADMINISTRATION OF NEBULISED RIBAVIRIN USING THE AIOLOS DEVICE

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Ribavirin is a broad-spectrum antiviral agent, which inhibits a wide range of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) viruses by disrupting viral protein synthesis. The current licensed Virazole® (ribavirin for inhalation) is for RSV, but is widely used in other viral infections. There have been difficulties following the discontinuation of the small particle aerosol generator (SPAG) and the transition to the AIOLOS nebuliser. There have been many issues vocalised regarding concerns of nursing staff over the delivery of ribavirin by nebulisation. These encompass both the technical aspects of the equipment and the safety considerations. The main aim was to create written guidelines supported by an educational DVD to ensure the standardisation of practice in the administration of nebulised Ribavirin whilst using the Aiolos device. The secondary outcome was to ensure parity and equity of the education delivered to all staff throughout the United Kingdom and create an opportunity for them to be deemed competent in this procedure so as to deliver optimum care to patients. A multidisciplinary team (MDT), of experienced ribavirin users which included healthcare professionals from hospitals across the United Kingdom, came together to evaluate and discuss all the relevant issues and by consensus developed a guidance document. The results of which consolidate best practice, and go someway to decrease the concerns of staff by addressing the myths surrounding the usage of ribavirin. The purpose of this these documents are to act as reference material for healthcare professionals who are being trained in the preparation and administration of the drug ribavirin by nebulisation. It is also intended as a reference for those who are deemed to have been competently trained. It has been widely recognised that there has been a demand for a document such as this to provide information on the use and environmental exposure to nebulised ribavirin. The success of the guidelines can be contributed to the collaborative approach of healthcare professionals who identified a need to address the inconsistencies with the delivery of this drug so as to ensure best practice nationally for both staff and patients.

1794

SUCCESSFUL GENERATION OF MYELOMA-SPECIFIC CYTOTOXIC T LYMPHOCYTES BY ALPHA-TYPE 1-POLARIZED DENDRITIC CELLS LOADED WITH APOPTOTIC ALLOGENEIC MYELOMA CELLS OF MATCHED SUBTYPES AGAINST AUTOLOGOUS MYELOMA CELLS

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In multiple myeloma (MM), it is not only impractical to obtain sufficient amount of autologous tumor cells as a source of tumor antigen in the clinical setting but it is also unsuitable for those with a lower tumor-burden status. In this study, we investigated the feasibility of cellular immunotherapy using autologous alpha-type 1-polarized dendritic cells (αDC1s) loaded with the UVB-irradiated allogeneic myeloma cells from other patients with the same subtypes, which could generate myeloma-specific cytotoxic T lymphocytes (CTLs) against autologous myeloma cell in patients with MM. Material and *Methods.* We generated autologous immature DCs from mononuclear cells of myeloma patients cultured with GM-CSF and IL-4 for 6 days. Their maturation was induced by adding the αDC1-polarizing cocktail composed of IL-1β, TNF-α , IFN-α, IFN-gamma and poly(I:C) and followed by loadedd with apoptotic allogeneic CD138+ myeloma cells irradiated with UVB (120 mJ/cm^2) as an tumor antigen. *Results.* αDC1s pulsed with allogeneic tumor antigen significantly increased the expression of several costimulatory molecules without the differences by those pulsed with autologous myeloma antigen. αDC1s showed high production of IL-12 during maturation and after subsequent stimulation of CD40L, but were not significantly affected by loading allogeneic myeloma antigens. Myeloma-specific CTLs against autologous CD138+ myeloma cells from MM patients were successfully induced by αDC1s loaded with allogeneic CD138+ myeloma cells in ELISPOT and JAM assay. In addition, there were no differences of CTL responses between pulsing autologous and allogeneic tumor antigen against targeting patient's myeloma cells. The specificity of the CTL response was demonstrated by the lowest level of IFN-gamma secreting cells against the NK-sensitive cell line K562. Pre-incubation of the target cells with anti-MHC class I or II monoclonal antibodies (mAbs) inhibited IFN-gamma release, indicating MHC class molecule-dependent cytotoxicity. *Conclusions.* Our data indicate that autologous DCs loaded with allogeneic CD138+ myeloma cells can generate potent myeloma-specific CTL responses against autologous myeloma cells and could be a highly-feasible and effective method for cellular immunotherapy in patients with MM.

1795

THE IMMUNOSUPPRESSIVE CAPACITY OF MESENCHYMAL STROMAL CELLS ISOLATED FROM MULTIPLE SCLEROSIS PATIENTS ON PHA-INDUCED T-CELL PROLIFERATION WAS SIGNIFICANTLY REDUCED

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Background. Autologous bone marrow multipotent mesenchymal stromal cells (MSCs) are being currently considered as a potential cell source for therapy of autoimmune diseases (AD), due to their immunomodulatory properties. MSCs have shown ability to suppress lymphocyte proliferation *in vitro* and possess immunoregulatory functions in other immune cells. Several mechanisms have been involved in the immunosuppressive effects of MSCs, including direct cell-to-cell contact and secretion of soluble factors. These properties of MSCs suggest a potential role of these cells in the induction of tolerance in AD and also provide a rational basis for their application in the treatment of multiple sclerosis (MS). However, it is unknown whether MSCs isolated from MS patients are normal or defective. Besides, there is no data concerning antiproliferative capacity of MS patients' MSCs. *Aim.* To evaluate the antiproliferative capacity of MSCs derived from MS patients and healthy bone marrow donors on allogeneic lymphocytes isolated from healthy individuals. *Methods.* MSCs cultures were derived from bone marrow aspirates from healthy donors (N=6) and MS patients (N=6). Mononuclear cells were separated by Ficoll-Hypaque density gradient and MSCs were isolated by plastic adherence. Peripheral blood mononuclear cells (PBMCs) separated from healthy individuals were co-cultivated with patients' or healthy donors' MSCs in the presence of phytohemagglutinin (PHA) at 37°C in a 5% CO2. We tested several MSCs:PBMCs ratios (1:2, 1:5, 1:10, 1:20, 1:50 and 1:100). After 5 days of co-culture, collected PBMCs were labeled with phycoerythrin-conjugated anti-CD3 monoclonal antibodies and T-cell proliferation was assessed by CFSE dilution method and the fluorescence was analyzed by flow cytometry. This study was approved by the Clinical Hospital of Ribeirão Preto ethics committee and an informed consent was signed by patients and controls before collection.

Table.

Table: Proliferation and proliferative inhibition percentages from CD3+T-cells co-cultivated with different ratios of patients' MSCs and healthy donors' MSCs.

CD3+ T-cells	PBMCs + PHA	MSCs: PBMCs 1:2	MSCs: PBMCs 1:5	MSCs: PBMCs 1:10	MSCs: PBMCs 1:20	MSCs: PBMCs 1:50	MSCs: PBMCs 1:100
patients P%	63.6±11.7	32.4±3.4	37.2±28.3	41.3±25.9	44.8±18.9	49.3±12.9	51.6±15.9
PI%	-	47.8±10.2	44.2±40.5	37.7±34.9	31.0±22.3	22.9±12.7	19.6±16.8
controls P%	71.9 ± 5.7	14.7±6.9	26.8±10,7	36.1±10.6	49.3±10.5	63.7±9.0	69.4±6.9
PI%	-	79.7±8,7	62.8±13.6	49.6±14.3	30.7±17.2	10.7±15.3	3.2 ± 9.2

MS: Multiple sclerosis; P%: Proliferation percentage; PI%: Proliferative inhibition percentage; PBMCs: Peripheral blood mononuclear cells; PHA: Phytohemagglutinin; MSCs: Multipotent mesenchymal stromal cells. The collected cells were labeled with PE-conjugated anti-CD3 monoclonal antibodies. The results were represented by mean ± std deviation from six experiments using MS patients' MSCs and healthy donors' MSCs.

Results. We observed that both MSCs, isolated from healthy donors and MS patients efficiently inhibited PHA-induced CD3+T-cell prolifer-

ation in a dose-dependent manner, as characterized by a decrease of CFSE peak generation number. However, MS patients' MSCs inhibited T-cell proliferation at lower levels than the inhibition obtained from healthy donors' MSCs. There were significant differences in proliferation mean (P) and proliferative inhibition (PI) percentages when comparing patients' MSCs (P:32.4±3.4; PI: 47.8±10.2) and healthy donors' MSCs (P:14.7±6.9; PI: 79.7±8.7) at ratio 1:2 (Table). The antiproliferative capacity of healthy donors' MSCs was significantly higher than patients' MSCs (P=0.005). There were no significant differences in T-cell proliferation inhibition from patients' and healthy donors' MSCs in the other tested ratios (1:5, 1:10, 1:20, 1:50 and 1:100). *Conclusions.* Both MSCs, isolated from MS patients and healthy donors, were able to inhibit PHA-induced T-cell proliferation in a dose-dependent manner. However, the immunosuppressive capacity of MSCs derived from MS patients was reduced compared to MSCs from healthy donors, once we observed significant differences in proliferation and proliferative inhibition percentages at ratio 1:2. These results suggest decreased and defective antiproliferative capacity of patients' MSCs on T-cell proliferation and therefore, more studies should be conducted before considering the use of MSCs from MS patients in autologous clinical settings.

1796

BIOLOGICAL ACTIVITY OF LONG-TERM CRYOPRESERVED PERIPHERAL BLOOD HEMATOPOIETIC STEM CELL HARVEST AND ITS ABILITY TO EXPAND CYTOKINE-INDUCED KILLER CELLS

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Background_Cytokine-induced killer (CIK) cells are a population of cytotoxic cells by *ex vivo* culturing peripheral blood mononuclear cells (PBMC) in the presence of different cytokines . There are no reports about if these cells are able to be inducted into cytotoxic CIK cells *ex vivo* after long-term cryopreserved. *Aims.* To observe whether cytotoxic CIK cells could be expanded from long-term cryopreserved PBSC harvest. *Methods.* Ten samples of PBSC which having been cryopreserved in liquid nitrogen for 3-6 years were used as test group and 7 fresh ones as control. CIK cells were derived from the RBC-removed PBSC harvest with cytokines: IFN-γ, CD-mAb and IL-2. On day 14, CIK cells were harvested to test viability and amplification. Proliferation ability of CIK cells was measured by calculation, phenotype of CIK cells was analyzed by flow cytometry and cytotoxicity of CIK cells against tumor cells was measured by MTT method. *Results.* CIK cells from the test group and the control group expanded 41.8 fold and 22.8 fold, respectively. The percentage of $CD8^+$ T cell increased after culture ($P<0.05$); while the purity of NKT cells ($CD3^+$ CD16 /56 +T cells) did not statistically increase. There was no significant difference between the two groups on the cytotoxicity against K562 cells. *Conclusion.* Cytotoxic CIK cells can be expanded from long-term cryopreserved PBSC harvest.

1797

PROSPECTIVE COMPARISON ON CARDIAC IRON AND LIVER IRON BY MR IN THALASSEMIA MAJOR PATIENTS TREATED WITH COMBINATION DEFERIPRON-DESFERRIOXAMINE VERSUS DEFERIPRON AND DESFERRIOXAMINE IN MONOTERAPY

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Background. Using T2* MR a randomized placebo controlled study from Sardinia demonstrated combination therapy with deferipron and desferrioxamine (DFP+DFO) significantly more effective than DFO in improving myocardial iron. One non-randomized study from Sardinia and one observational study from Greece seem to confirm for DFP+DFO therapy the most rapid clearance of cardiac iron. No data are available in literature about prospective comparisons on cardiac iron and function and liver iron in TM patients treated with DFP+DFO *vs.* DFP and DFO in monotherapy. *Aim.* The aim of this multi-centre study was to assess prospectively in a large clinical setting the efficacy of the DFP+DFO *vs.* DFP and DFO in TM patients by quantitative MR. *Methods.* Among the first 739 TM patients enrolled in the MIOT (Myocardial Iron Overload in Thalassemia) network, 253 patients performed a MR follow up study at 18±3 months according to the protocol. We evaluated prospectively the 43 patients treated with DFP+DFO *vs.* the 30 patients treated with DFP and the 66 patients treated with DFO between the 2 MR scans. Myocardial and liver iron concentrations were measured by T2* multislice multiecho technique. Biventricular function parameters were quantitatively evaluated by cine images. *Results.* The doses of the combination treatment were DFP 66±23 mg/kg/d for 6±1 d/w and DFO 41±7 mg/kg/d for 4±1 d/w, the dose of DFP was 73±16 mg/kg/d, DFO was 41±7 mg/kg/d for 5.5 d/w. Excellent/good levels of compliance were similar in the 3 groups (DFP+DFO 91% *vs.* DFP 97% *vs.* DFO 92%; P=0.76). Among the patients with no significant myocardial iron overload at baseline (global heart T2*±20 ms), there were no significant differences between groups to maintain the patients without myocardial iron overload (DFP+DFO 90% *vs.* DFP 100% *vs.* DFO 100%; P=0.053). Among the patients with myocardial iron overload at baseline (global heart T2*<20 ms), in all groups there was a significant improvement in the global heart T2* value (DFP+DFO P=0.0001, DFP P=0.001 and DFO P=0.003), in the number of segment with a normal T2* value (DFP+DFO P=0.0001, DFP P=0.031, DFO P=0.0001) and in the right global systolic function (DFP+DFO P =0.002, DFP P=0.031, DFO P=0.045). The improvement in the global heart T2* was significantly different among groups (mean difference global heart T2* DFP+DFO 6.6±6.5 ms, DFP 10.7±7.2, DFO 3.6±5.4; P=0.009). The improvement in the global heart T2* was significantly higher in the DFP+DFO *vs.* the DFO group (P=0.017), but it was not significantly different in the DFP+DFO *vs.* the DFP group (P = 0.36) (see the Figure). The changes in the global systolic bi-ventricular function were not significantly different among groups. In patients with liver iron overload at baseline (liver T2*<5.1 ms), the change in the liver T2* was not significantly different among groups (mean difference liver T2* DFP+DFO 2.9±4.7 ms, DFP 2.3±5.8, DFO 2.9±4.9; P=0.91). *Conclusions.* prospectively in a large clinical setting over 15 months in TM patients combined therapy DFP+DFO confirmed superior reduction in myocardial iron in comparison to DFO, but no significant differences were found *vs.* DFP monotherapy.

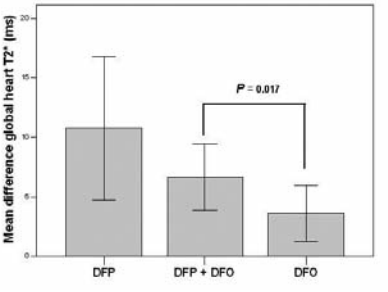

Figure.

1798

EFFECT OF DEFERASIROX ON RENAL HAEMODYNAMICS IN PATIENTS WITH BETA-THALASSAEMIA: FIRST INTERIM ANALYSIS

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Background. Previous deferasirox studies have shown that some patients experience dose-dependent, non-progressive increases in serum creatinine (SCr), most frequently at doses of ≥30 mg/kg/day. Although the mechanism of these increases is unclear, the hypothesis is that changes are caused by a decreased glomerular filtration rate (GFR) due to a pharmacological effect of deferasirox on renal haemodynamics. *Aims.* To estimate the short-term effect of deferasirox 30 mg/kg/day on renal haemodynamics by measuring changes in the markers of renal function, in particular GFR, renal plasma flow (RPF) and filtration fraction (FF) and assessing their relationship with SCr, estimated creatinine clearance (CrCl) and serum ferritin (SF) in iron-overloaded patients with β-thalas-

saemia. *Methods.* This study enrolled deferasirox-naïve β-thalassaemia patients aged ≥18 years receiving transfusions every 2-5 weeks (iron intake ≥0.25 mg/kg/day), a transfusion history of ≥20 units of packed red blood cells, and baseline SF ≥500 ng/mL or liver iron concentration (LIC) ≥2 mg Fe/g dry weight (dw). Patients were excluded if they had baseline SCr >upper limit of normal (ULN), estimated CrCl <60 mL/min, urinary protein:creatinine ratio >0.5 mg/mg, history of nephrotic syndrome, or treatment with drugs affecting renal parameters. Patients received deferasirox 30 mg/kg/day for 8 weeks, followed by 2 weeks of washout. GFR and RPF were measured using chromium-labelled ethylenediaminetetraacetic acid (51Cr-EDTA) and 123ortho-iodohippurate (123I-OIH). All parameters were assessed at baseline, 2, 8 and 10 weeks. Descriptive statistics were provided. This planned interim analysis reports data after 50% of patients have completed the 10-week core study. *Results.* Ten patients were enrolled (mean age 34.3 years; 6:4 male:female); one discontinued due to voluntary withdrawal on day 14. Median baseline SF was 2232 ng/mL and mean LIC 9.8±6.7 mg Fe/g dw. Mean GFR (112.5±18.1 mL/min), RPF (608.6±97.9 mL/min) and FF (0.2±0.03) were normal at baseline. Mean actual deferasirox dose over 8 weeks was 29.6±1.8 mg/kg/day. After 8 weeks of treatment mean GFR decreased by 15% (90% CI -21%, -10%) and RPF by 15% (90% CI -21%, -9%) (Figure), which was paralleled by a 17% decrease in estimated CrCl. Median SF decreased by 31 ng/mL. GFR, RPF and estimated CrCl returned to near baseline after 2 weeks' washout. Mean FF showed non-significant fluctuations, while mean SCr increased slightly (22%) then returned to near baseline after 2 weeks' washout. There were no significant relationships or clear trends between absolute changes in GFR, RPF and FF, and either SCr, CrCl or SF. Trough concentrations of deferasirox showed no substantial differences from weeks 1 to 8. Investigator-assessed drug-related adverse events (AEs) included rash (n=2), upper abdominal pain (n=1) and pruritus (n=1). There were no deaths, serious AEs or discontinuations because of AEs. *Conclusions.* Deferasirox appears to produce a mild effect on renal haemodynamics, reflected by a decrease in RPF, leading to a decrease in GFR; this was reversible after drug discontinuation. Longer observation is required; this study will continue to assess the effects of deferasirox treatment on renal haemodynamics up to 2 years.

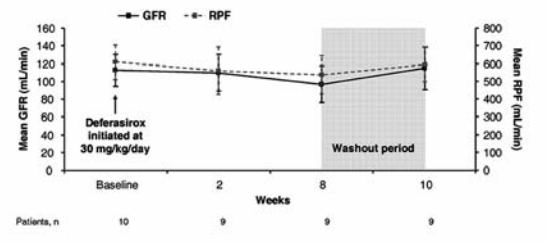

Figure. Mean (SD) GFR and RPF level in patients ith b-thalassemia.

1799

HYPERTONIC CRYOHEMOLYSIS (CH) IS A USEFUL TEST FOR THE DIAGNOSIS OF HEREDITARY SPHEROCYTOSIS (HS): DETERMINATION OF HIGH SENSITIVITY AND SPECIFICITY VALUES WITH A SLIGHTLY MODIFIED METHOD

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Background. CH is a laboratory test developed a few years ago for diagnosing HS, based on the special susceptibility of spherocytes to cooling while suspended in hypertonic solutions. Its usefulness seems to be similar to eosin-5'-maleimide flow cytometry and osmotic fragility tests. However, its usual utilization is not widely accepted because of controversial sensitivity and specificity results reported by several authors. *Aims.* A) To determine specificity and sensitivity of CH test for the diagnosis of HS. B) To assess the usefulness of CH for study of anemias. C) To visualize morphologic changes occurring in red blood cell as CH is being performed. *Methods.* 228 normal controls and 73 anemic patients (HS 53, iron-deficiency 5, Beta thalassemia 8, hemoglobin S/Beta thalassemia 1, hereditary persistence of hemoglobin F 1, aplastic anemia 1, autoimmune hemolytic anemia 1) were studied. CH test was performed according to the method described by Streichman and Gescheidt, slightly modified. Differences with the original procedure were the use of manual shacking instead of vortex and a sucrose concentration slightly lesser than the original method (0.6 M *vs.* 0.7 M, respectively). Scanning electron microscopy (SEM) was carried out as CH was performed in one HS patient and one normal control. Between-groups comparisons were done using the student's t test. ROC analysis was used to determine specificity and sensitivity of CH. *Results.* Comparison between groups showed that HS patients had CH results significantly lower than non-spherocytic anemias and normal controls (P<0.0001) (Table). Non-spherocytic anemia showed significantly higher CH results than controls (P:0.03). Sensitivity and specificity were firstly established in a population composed by 106 normal controls and 19 HS patients; the established cut-off value was 2.8% (sensitivity 78%, specificity 96%). Thereafter, the analysis of a second cohort of 117 normal controls and 31 HS patients validated the previously established cut-off value (sensitivity 77%, specificity 95%). The use of ROC curves applied to the total number of studied cases established the following definitive values: cut-off value 2.8%, sensitivity 79.2%, specificity 95.0%, AUC 0.909. SEM allowed visualization of the lack of plasticity of spherocytes when exposed to sudden changes of temperature in a hypertonic medium. *Conclusions.* Reference values in our study are noticeably lower than those reported by other authors; however, they were established on a large number of samples, and were subsequently validated. We suggest that our modifications to the original method could explain these differences. The main advantages of CH over other HS diagnostic tests are the smaller volume of blood sample required, the shorter processing time, no requirement of specific equipment, and no need for use of simultaneous normal controls.

Table.

Group	Cryohemolysis (%)	
	Median (range)	Mean ± SD
Normal controls	1.39 (0.60 – 4.27)	1.51 ± 0.58
Hereditary Spherocytosis	8.45 (0.99 – 34.64)	9.38 ± 8.04
Non-spherocytic anemias	1.14 (0.59 – 2.23)	1.22 ± 0.45

1800

SIGNIFICANT SENSORINEURAL HEARING LOSS IN OMANI PATIENTS WITH SICKLE CELL DISEASE

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Background. Sickle cell disease (SCD) is characterized by chronic hemolytic anemia, increased susceptibility to infections, and intermittent episodes of vascular occlusion and end-organ damage. Neurological symptoms are frequent and the potential for auditory damage is not unexpected. However, the incidence of subjective hearing impairment among sickle cell disease subjects is very low. *Aim.* To study the prevalence and pattern of hearing loss in Omani patients with SCD.

Table. Significant Hearing loss in SCD patients.

Frequencies tested	SCD patients [n=49] Mean		Control Subjects [n=31] Mean		Student t' test [SCD patients; Rt v/s Lt] p value	Student t' test [SCD patients v/s Controls] p value	
	Rt. Ear	Lt. Ear	Rt. Ear	Lt. Ear		Rt. Ear	Lt. Ear
250	18.16	16.42	13.22	12.41	0.48	0.03*	0.06
500	17.55	17.44	14.67	14.35	0.97	0.2	0.3
1000	16.12	16.44	12.91	12.41	0.92	0.16	0.39
2000	17.14	16.93	12.25	11.45	0.94	0.04*	0.08
4000	18.97	19.48	13.54	12.58	0.88	0.03*	0.11
8000	24.28	26.12	16.45	16.12	0.67	0.011*	0.07

* p <0.05; Significant

Methods. A prospective case control study of SCD patients attending the outpatient department and control subjects from age and sex matched normal volunteer blood donors were conducted after an informed consent and Medical Ethics Committee approval. Tympanometry and diagnostic audiometry were performed in all cases studied. *Results.* Forty-nine SCD patients (15 males, 30.61%) aged 16-43 years with a mean age of 29.93 years +6.8 and 31 controls (10 males, 32.25%) aged 15-39 years with a mean age of 25.03 years + 7.8 were enrolled in this study. The average hearing thresholds of SCD patients were consistently higher than controls in all frequencies tested in both right and left ears, with preponderance for the right ear and females. [Table] Of the 98 ears of SCD patients tested 11.22% had sensorineural hearing loss [SNHL]. 3 patients had bilateral SNHL; additionally 2 and 3 cases respectively had SNHL in left and right ears. All the controls had hearing thresholds within normal limits. *Summary/Conclusions.* The study reveals a significant incidence of SNHL in SCD patients although the patients were clinically asymptomatic. The hearing loss was worse in the right ear and has a female preponderance. Furthermore, the hearing loss was also more severe at the higher threshold i.e 8000. We hope that more aggressive primary and secondary prevention and adequate treatment of sickle cell crisis would reduce, if not eliminate the hearing loss found in SCD patients. Regular audiometric assessment, counseling and rehabilitation of these patients with hearing aids are recommended.

1801

T2*MRI - AN EFFECTIVE TOOL TO INCREASE CHELATION COMPLIANCE IN THALASSEMIA MAJOR

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Background. Evaluation of cardiac iron load by T2* magnetic resonance imaging has become a standard tool in the management of beta thalassemia, and is used to monitor cardiac and liver iron load. *Aims.* The aim of this analysis was to see if regular monitoring by T2*MRI had any effect on chelation compliance in patients with thalassemia major. *Patients.* Inclusion criteria were a) data for cardiac T2*, liver T2* at baseline and at 3years;b) S ferritin for 3 years preceding and 3 years after instituting T2*; c) chelation with combined chelation (desferal + deferiprone) or deferiprone monotherapy (where the S ferritin was <500 ng/mL) with no marked change in prescribed chelation therapy during the 6 years of analysis (3 yrs pre- and 3years post- T2*) . T2* was repeated at intervals of 3-12 months. The patients were stratified into 3 risk groups according to cardiac T2*: 0-10ms- high risk, 10.1- 20ms - moderate risk, and >20 ms - low risk. In addition, S. ferritin for the 3 years preceding instituting T2* was compared to the 3 years post T2* availability. *Intervention.* The T2* images obtained were shown to patients (and parents where applicable) and discussed fully. Counseling regarding iron chelation was intensified if either the heart or the liver was heavily loaded. Patients with 'good 'or improving T2* results were given positive feedback. *Results.* Data was available for 60 patients from a single centre in the Sultanate of Oman. Full cohort analysis showed no significant change in cardiac iron load over the 3 year study period, (mean 22.1ms to 24.7ms). However, when analyzing the sub groups, patients in the high and moderate risk groups showed a significant improvement in cardiac T2* (P=0.006 and 0.04 respectively), with no significant change in the low risk group. Analysis of S ferritin for the whole cohort in the 3 years preceding T2* showed no significant improvement, (mean 2561ng/mL, 2655ng/mL, and 2623ng/mL, P=0.9). Although the improvement in S ferritin in the following 3 years only approached significance (P=0.06), there was a significant improvement of liver iron load as measured by T2* across the whole cohort over the 3 years post T2* implementation (P=0.03). *Conclusions.* We attribute the improvement in iron load parameters after instituting T2* to increased awareness and vigilance on the part of both physicians and patients. This was particularly important in those patients who were found to have discordance between S ferritin and cardiac iron load. Patients can clearly see improvement (or deterioration) on their own T2* images and we think that this, combined with more intensive, focused counseling has led to improved compliance.

1802

PREVALENCE OF PULMONARY HYPERTENSION IN SICKLE CELL DISEASE PATIENTS (EXPERIENCE FROM TWO CENTERS IN EASTERN SAUDI ARABIA) DO WE REALLY NEED HYDROXY UREA?

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Abstract. Pulmonary hypertension is one of the leading causes of morbidity and mortality in adult sickle cell disease, with a prevalence of 20% to 40%. The presentation of (SCD) in eastern province Saudi Arabia is variable in part due to the heterogeneous population and certain demographic criteria of that region. *Aim of the work.* to evaluate the prevalence of pulmonary hypertension in patients from this region and correlate our findings with the well documented characters of this patients population, comparing those on hydroxyurea treatment and those with out it. *Methods.* Doppler echocardiography was performed in 100 consecutive patients with SCD; 58 men and 42 women. Mean age was 32±6.Pulmonary hypertension was prospectively defined as a tricuspid regurgitant jet velocity of at least 2.5 m per second, and graded accordingly. The results showed that out of 100 patients, 15 had tricuspid regurgitation, with pulmonary systolic pressure ranging from36 to 60mm Hg (mean of 43.3±4.8 mmHg) which was well correlated with markers of hemolysis; including LDH, and reticulocytic count. Pressure correlation with hemoglobin F concentration revealed an inverse relationship, and higher levels correlated with lower TRJV values (P<.001). Yet lower arginase activity was well correlating with higher TRJV. Another documented point in our study was those patients who were on hydroxyurea; 45 patients, showed no significant difference as regard TRJV when compared with patients off hydroxyurea. (Mean TRJV was 2.57m/s and 2.6m/s respectively). *In summary.* patients with SCD from eastern province Saudi Arabia have special characters with pulmonary hypertension of less prevalance possibly due to higher HbF levels.

1804

MILD HEMOLYTIC ANEMIA, PROGRESSIVE NEUROMOTOR RETARDATION AND FATAL OUTCOME: A DISORDER OF GLYCOLYSIS, TRIOSE- PHOSPHATE ISOMERASE DEFICIENCY

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Introduction. Triose-phosphate isomerase (TPI) deficiency is a rare autosomal recessive multisystem disorder of glycolysis, characterized by decreased enzyme activity in all tissues, which is accompanied by the elevation of dihidroxyacetone phosphate (DHAP) level in erythrocytes. Homozygotes manifest congenital hemolytic anemia, increased susceptibility to infection, cardiomyopathy and progressive neuromuscular impairment and fatal outcome in early childhood. *Case report.* A 2 month-old male infant presented with jaundice and pallor. D.Coombs negative hemolytic anemia with normal osmotic fragility, G-6PD, and PK levels was diagnosed. The history revealed hyperbilirubinemia and anemia (Hb:6 g/dL) requiring phototherapy and transfusion since first post-natal day. Blood groups of mother and the infant did not confirm immune hemolytic anemia. The parents reported that their first baby also had hemolytic anemia, neuromotor retardation and at the age of 21 month he was found dead. Screening for inborn errors of metabolism was not diagnostic. There was no consanguineous marriage. During follow-up hemolytic anemia was mild and no transfusion was required. Although the baby gained excessive weight neuromotor development was retarded. There were intermittent tremor and sweating episodes. On admission at the age of 14 month, body weight was 15 kg (>97 pers.), height 83 cm (75-90 pers.) and head circumference 51 cm (>97 pers.). The patient had normal features with no jaundice or pallor. He had poor head control, could not sit and had difficulty in swallowing in the last week. Respiratory and heart rate were increased to 48/min and 152 beats/ min, respectively. Blood counts were: WBC 20600/mm^3, ANC 12400 /mm^3, PLT 345000/mm^3, MCV:111 fL and reticulocyte count was 277 000/mm^3 Peripheral blood smear showed normal erythrocyte morphology and rare basophilic stippling. T.bilirubin 4.6 mg/dL, d.bilirubin 0.6 mg/dL, ALT 33 IU/L, AST 53 IU/L, venous blood PH 7.29, PCO2 62, HCO3 29 (respiratory acidosis). Direct chest radiogram was normal. Echocardiogram showed EF 63%, small muscular ventricular septal defect, patent foramen ovale. Brain natriuretic peptide level was elevat-

ed (337 pg/mL), cardiac troponin I and creatin kinase MB levels were normal. The patient was entubated and ventilatory support was introduced. After a medline search, TPI deficiency was suspected. A highly elevated DHAP was found in patient's erythrocytes and DNA analysis showed a homozygous missense mutation in TPI gene (DNA level: c.315G>C, protein level:p.(Glu 105Asp). The patient lived for 3 months under ventilatory support and died. *Conclusion.* In infants with mild hemolytic anemia and neuromotor retardation this rare glycolytic pathway defect must be suspected. The defect causes impairment of energy metabolism and/or formation of toxic protein aggregates. Although no effective therapy is available, genetic confirmation of diagnosis gives chance for prenatal diagnosis and genetic counseling. *Acknowledgement.* Thanks to Prof.Dr.C.Jakobs and Dr.Gajja.S.Salomons from Dept. Clinical Chemistry, Metabolic Unit from VU University Medical Center in Amsterdam for erythrocyte DHAPanalysis and genetic study.

1805

DEFERASIROX EFFECTIVELY REMOVES CARDIAC IRON IN β-THALASSAEMIA PATIENTS WITH MYOCARDIAL SIDEROSIS PREVIOUSLY CHELATED WITH DEFEROXAMINE MONOTHERAPY OR DEFEROXAMINE-DEFERIPRONE THERAPY

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Background. In the cardiac substudy of the deferasirox EPIC trial, patients who entered the extension study received deferasirox treatment for up to 2 years. All patients enrolled in the extension study were previously chelated, either with deferoxamine (DFO) monotherapy or DFO-deferiprone (DFP) therapy. The efficacy of monotherapy deferasirox was assessed in patients previously chelated with DFO monotherapy or DFO-DFP therapy. *Aims.* To evaluate the effect of 2 years of treatment with deferasirox on cardiac iron, liver iron concentration (LIC) and serum ferritin (SF) in β-thalassaemia patients stratified by prior chelation regimens. *Methods.* Eligible patients were aged ≥10 years with myocardial T2* >5-<20 ms by cardiovascular magnetic resonance (CMR), left ventricular ejection fraction (LVEF) ≥56%, SF >2500 ng/mL, MR (R2) LIC >10 mg Fe/g dry weight (dw), and a lifetime minimum of 50 transfused blood units. All were previously chelated with DFO monotherapy or DFO-DFP therapy. Deferasirox was initiated at 30 mg/kg/day and increased to 40 mg/kg/day by the time patients had entered the extension. Dose decreases were allowed for safety reasons. *Results.* Overall, 95 patients had MRI assessments available at baseline and after 18 and/or 24 months of deferasirox treatment. Sixty-three patients (66.3%) had previously received DFO monotherapy; the mean age of these patients was 18.6±6.3 years. Thirty-two patients (33.7%) had previously received DFO-DFP therapy; mean age was 24.3±7.8 years. The mean duration of prior chelation therapy for the DFO monotherapy group was 12.0±6.3 years, and that for patients having received DFO-DFP as part of their previous chelation was 16.8±8.2 years. Average actual deferasirox dose during treatment was 34.2±5.3 and 35.2±4.0 mg/kg/day, respectively. Geometric mean myocardial T2* significantly increased by 28% in the prior DFO monotherapy group (11.8 to 15.2 ms, P<0.0001), and by 35% in the prior DFO-DFP group (10.4 to 14.0 ms, P<0.0001). In the prior DFO monotherapy group, 50/63 patients (79.4%) had an improvement in T2* of >4%; this proportion in the prior DFO-DFP group was 22/32 (68.8%). Mean LVEF increased by 1.2±4.5% (P=0.046) in the prior DFO monotherapy group; the change in the prior DFO-DFP group was not significant (-1.7±5.8%, P=0.102). In the prior DFO monotherapy group, both mean LIC and median SF were significantly reduced from baseline by 9.6±12.7 mg Fe/g dw and 2227 ng/mL, respectively (P<0.001; based on last-observation-carried-forward analysis); similar data were observed in the prior DFO-DFP group (12.4±12.5 mg Fe/g dw and 2559 ng/mL; P<0.001). *Conclusions.* Two years of deferasirox treatment led to significant improvements in cardiac T2*, LIC and SF, irrespective of whether patients had previously received DFO monotherapy or DFO-DFP therapy. This suggests that heavily iron-overloaded patients can be effectively switched to once-daily deferasirox from other chelation regimens containing DFO, which may be cumbersome to administer and therefore may lead to poor compliance and, potentially, suboptimal efficacy.

1806

IMPACT OF DIFFERENT I.V. IRON COMPOUNDS ON HEMODYNAMIC AND INFLAMMATORY RESPONSE, MARKERS OF OXIDATIVE STRESS AND IRON DEPOSITION

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Background. Iron deficiency anemia associated with different conditions has been effectively managed with intravenous (I.V.) iron. I.V. iron preparations may induce different physiological reactions depending on their composition and the stability of the complexes. Therefore, the effects of five I.V. iron preparations on hemodynamic and inflammatory response, oxidative stress markers and iron deposition were examined in a nonclinical rodent model. *Methods.* Ten rats per group were treated with ferric carboxymaltose, high-molecular-weight (HMW) iron dextran, low-molecular-weight (LMW) iron dextran, ferric gluconate, iron sucrose or saline solution as control. Five I.V. doses of iron (40 mg iron/kg) or saline were administered every 7 days over 4 weeks (days 0, 7, 14, 21 and 28). Blood samples were collected 24h after I.V. iron treatment (days 1, 8, 15, 22 and 29). Urine was collected for 24h at the same time period as blood sampling. Rats were sacrificed 24h after the last I.V. iron dose. Blood-free liver, heart and kidneys of each rat were removed for analysis of oxidative stress markers, microscopy and immunohistochemical evaluation. Investigators were blinded to the treatment group. *Results.* At all assessments after initial treatment, systolic blood pressure was significantly reduced in the LMW dextran group compared to all other groups, whereas proteinuria was significantly increased in the ferric gluconate group compared to all other groups (P<0.01). Compared to ferric carboxymaltose and iron sucrose, ferric gluconate was also associated with a significant reduction of creatinine clearance on days 15, 22 an 29 (P<0.01). Inflammatory cytokines TNF-α and IL-6 showed highest levels in the liver, heart and kidney of rats treated with ferric gluconate (P<0.01 *vs.* all groups). Both cytokines were also significantly higher in the iron dextran groups compared to ferric carboxymaltose, iron sucrose and saline. Blood levels of liver enzymes alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) showed the same pattern on days 1, 8 and 29; significantly highest in the ferric gluconate group and increased in both iron dextran groups compared to the other groups (P<0.01). Oxidative stress markers malondialdehyde (TBARS) and catalase were significantly increased in liver, heart and kidney homogenates of rats in the ferric gluconate group (P<0.01). CuZn-superoxide dismutase and glutathione peroxidase activity were increased and the ratio of reduced to oxidized glutathione decreased in the ferric gluconate and two dextran groups (P<0.01), whereas ferric gluconate was associated with largest deviations from control. In line with the oxidative stress markers, homogenates of organs from ferric gluconate treated rats showed significant amounts of iron (Prussian blue staining) not matched by high ferritin levels. Highest ferritin levels were measured in the ferric carboxymaltose and iron sucrose group. *Summary/Conclusions.* This study confirms that different I.V. iron preparations can result in different hemodynamic and functional response. Increased markers of inflammation and oxidative stress in vital organs of rats treated with HMW or LMW iron dextran or ferric gluconate suggest a less favorable safety profile for these compounds than for ferric carboxymaltose and iron sucrose.

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1807

PLATELET FUNCTION ALTERATIONS AND THEIR RELATION TO P-SELECTIN (CD62P) EXPRESSION IN CHILDREN WITH IRON DEFICIENCY ANEMIA

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Background. The CD62P antigen (P selectin) is an integral membrane protein of platelets. Upon platelet activation, the CD62P antigen is expressed on the surface of the activated platelets. It has been demon-

strated the existance of chronic platelet activation associated with the increased fraction of platelets carrying the platelet activation markers, CD62P and CD63, in thalassemic patients. At the other hand, there have been reports demonstrating that iron deficiency anemia may cause platelet aggregation dysfunction and this can be reversed by iron therapy. Those studies made us think that there may be a relationship between body iron and platelet activation markers. Increased iron load may cause to increase in expression of activation markers in thalassemic patients; however, iron deficiency may result in diminished expression of activation markers leading to platelet aggregation dysfunction in children with iron deficiency anemia. *Aims.* To investigate the alterations of platelet functions and their association with platelet activation marker (p-selectin; CD62P) expression in children with iron deficiency anemia. *Patients and methods.* Hemoglobin, erythrocyte indexes (MCV and RDW), serum levels of iron, transferrin and ferritin, platelet aggregation tests (with ADP, collagen and ristocetin), invitro bleeding time (with PFA 100), and CD62P expression were evaluated in fasting blood samples of 19 children with iron deficiency anemia and 20 children without anemia. CD62P expression was detected by flow cytometry in normal and 5μM ADP-activated platelets. *Results.* Mean values of hemoglobin, MCV, RDW, serum iron and ferritin were significantly different in patient group as expected. However, there was no difference between two groups in terms of age, gender, mean platelet counts and serum transferrin levels (Table 1). Mean closure times (either with ADP or epinefrin) were longer in patient group than control. In platelet aggregation tests, mean values of maximum aggregation times by ristocetin, ADP and collagen were also more prolonged in patient group. However, maximum aggregation rates (amplitude) by ristocetin and ADP were significantly higher in patients. These findings suggest that platelet aggregation and adhesion have been delayed in children with iron deficiency anemia. CD62P expressions were significantly higher on activated platelets of patient group although they were similar in both groups before activation by ADP (Table 1). *Conclusion.* Invitro bleeding time (closure time) and maximum aggregation time of the platelets get prolonged in children with iron deficiency anemia. However, those are not associated with CD62P expression on platelet surface. ADP activation results in more prominent increase of CD62P expression on platelets of the iron deficient cases.

Table 1. The age, gender and laboratory values of the patient and control group.

	Patient (n:19)	Control (n:20)	p
Age (year); median (min-max)	6 (1–16)	4,5 (1–15)	0,81
Gender (F/M)	19 (6/13)	20 (6/14)	0,91
	Mean±SD	Mean±SD	
Platelet ($x10^9$/L)	448 ±199	402±174	0,45
Hemoglobin (g/dL)	6,68±1,97	12,76±1,40	**0,000**
MCV (fL)	63,01±5,80	81,33±5,78	**0,000**
RDW (%)	20,56±2,54	14,34±2,05	**0,000**
Serum iron (μg/dL)	15,47±5,91	50,75±25,65	**0,000**
Ferritin (ng/mL)	4,82±3,16	70,72±47,51	**0,000**
CD62P (%)	21,41±19,72	16,24±17,40	0,28
aCD62P (%)	31,43±21,17	20,50±21,78	**0,04**
PFA 100 (Col/ADP) (sec)	103,58±25,09	85,50±19,74	**0,019**
PFA 100 (Col/Epinefrin) (sec)	142,70±57,77	108,60±30,46	**0,028**
Ristocetin amplitude (%)	84,00 ±16,60	63,84±25,51	**0,008**
Ristocetin MAT (sec)	4,4±1,1	3,5±0,6	**0,004**
ADP amplitude (%)	72,00±28,94	53,78±24,79	**0,047**
ADP MAT (sec)	4,1±2	3,4±0,3	**0,007**
Collagen amplitude (%)	69,82±25,77	67,47±25,71	0,78
Collagen MAT (sec)	4,1±0,4	3,6±0,3	**0,002**

MAT: Maximum aggregation time

1808

USE OF CAPILLARY BLOOD TO PERFORM THE EOSIN-5'-MALEIMIDE FLOW CYTOMETRY (EMA-FC) TEST: RESULTS ARE COMPARABLE TO VENOUS BLOOD

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Background. Blood volume required to perform usual diagnostic laboratory tests may be hard or even not feasible to collect in neonates or infants with hemolytic anemia, due to either difficulties inherent to the sampling procedure or to the inconvenience of withdrawing large blood volumes in small babies. As a consequence, etiological diagnosis of the anemia may be delayed several weeks or months. The EMA-FC is a very useful test for the diagnosis of HS which allows getting results within 2 hours. The use of capillary blood samples to perform the test has not been reported in the literature. *Aims.* To assess the reliability of capillary blood for performing EMA-FC test. *Methods.* 31 pairs of capillary and venous blood samples belonging to 19 normal controls and 12 patients with anemia of different etiologies were simultaneously collected by venopuncture in tubes with EDTA and by digital puncture in heparinized microhematocrit tubes. EMA-FC test was simultaneously performed for both samples within 48 hours from sampling. Either the content of a microhematocrit tube or 30 μL of venous blood were washed three times with phosphate saline buffer (PSB); 5 μL of the red cell pellet were added to 5 μL of EDTA (0.5 mg/mL) and incubated 15 minutes at room temperature. Red blood cells were washed three times with PSB and suspended in 1 mL of PSB; 10000 events were acquired to determine the percentage of decrease of geometric mean fluorescence in FL-1 channel (MCF) and the increase of the coefficient of variation (CV). For the statistical analysis, the t student test for paired data and the Bland Altman graphic method were used. A p value less than 0.05 was considered significant. *Results.* No significant difference between venous and capillary blood samples was found (Table). *Conclusion.* Our results suggest that the use of capillary or venous blood makes no difference to the EMA-FC test *Results.* The use of capillary blood would allow an early etiological diagnosis in neonates and infants.

Table.

Blood sample	MCF (mean ± SD)			CV (mean ± SD)		
	Normal controls	Anemic patients	Total	Normal controls	Anemic patients	Total
Venous	105.9 ± 14.2	101.0 ± 9.0	104.0 ± 12.5	23.9 ± 6.7	29.8 ± 9.7	26.2 ± 7.9
Capillary	103.1 ± 13.4	101.4 ± 8.3	102.4 ± 11.6	23.9 ± 6.2	29.6 ± 8.1	26.1 ± 7.4
p	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

1809

GROWTH AND PUBERTY STATUS IN THALASSEMIA MAJOR AT ZAGAZIG UNIVERSITY HOSPITAL - EGYPT

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Introduction. Present transfusional regimen protocols for thalassemic patients have increased the life expectancy of patients with β- thalassemia major but caused a progressive iron overload that can be prevented or limited only by appropriate iron chelation. As a result of iron overload those patients develop endocrine abnormalities, cardiac failure and hepatic cirrhosis. Short stature and hypogonadism are extremely frequent in patients with thalassemia. *Aim of the work.* To evaluate the status of growth and puberty in patients with β- thalassemia major in pediatric hematology unit at Zagazig University Hospitals. *Patient and method.* One hundred patients with β- thalassemia major aged from 8-20 years old were enrolled in this study. They were classified according to regularity of iron chelation therapy into two groups (13 patients with regular chelation and 87 patients irregularly chelated). The following data were recorded in: history taking, age, sex, weight, height, serum ferritin levels and pubertal evaluation according to tanner score were done. *Results.* short stature was presented in 76% of our patients. Short stature in thalassemic patients is more apparent with increase in the age (92% of patients above 14 years old, 76% of patients aged from 10-14 years and 55% of patients younger than 10 years). There is a significant increase in height among patients with regular iron (141.1±7.4 cm) in comparison to irregular chelated patients (133.5±12.1 cm) with P value 0.025. Delayed puberty was present in 65% of patients (67.2% of males and 61.5% of females). The decrease of tanner among irregular chelated patients in comparison to regular is statistically insignificant. *Conclusion.* Compliance to iron chelation therapy is very low (13%). Growth retardation is one of common complications with significant correlation with age and ferritin level. Delayed puberty is another common complication with no correlation to ferritin levels or sex.

1810

EVALUATION OF IRON CHELATION THERAPY IN β-THALASSEMIA MAJOR PATIENTS IN EAST DELTA OF EGYPT

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Background. Beta Thalassemia is the most common chronic hemolytic anemia in Egypt. Desferrioxamine given by regular subcutaneous infusion is the standard iron chelator in these patients. However, failure of compliance is the major problem interfering with its regular use. *Aims.* We aimed to evaluate the efficacy and safety of alternating deferiprone and desferrioxamine in reducing transfusional iron overload compared to either drug alone and to assess the associations between compliance, complications and ferritin levels in thalassemic children in east delta of Egypt. *Methods.* Retrospective - Prospective cohort study was carried out on 150 transfusion dependant thalassemic children who are on irrigular desferrioxamine chelation therapy. Patients were randomized into three groups (50 patients in each group) according to iron chelation therapy regimen: Group I received deferiprone for 5 days/week alternating with desferrioxamine for 2 days/week, group II received daily deferiprone only and group III received desferrioxamine only 5 days/week. Efficacy, safety and tolerance of different chelation regimens were assessed by periodic assessment of all relevant clinical and laboratory data. *Results.* There was highly significant reduction in serum ferritin levels after chelation therapy in all studied groups. The reduction was significantly higher in group I and group III than group II especially in compliant patients. Patients of group II and group I were more compliant to chelation therapy than patients of group III. Regarding disease complications, hypogonadism and growth impairment were the commonest disease complications in our study followed by osteoprosis and osteopenia. Complications were higher in non compliant patients and those with higher serum ferritin levels. Regarding side effects of the chelators, gastrointestinal upset was the commonest complication in group II, local reactions were the commonest in group III and both complications were lower in group I. *Summary/Conclusions.* Dual chelation therapy regimen of alternating desferrioxamine and deferiprone is more effective, less toxic and well tolerated in comparison to either drug alone.

1811

THYROID DYSFUNCTION AND ANEMIA DURING INTERFERON THERAPY IN VIRUS C HEPATITIS

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Background. Thyroid disease is a frequent side effect of interferon (INF) - therapy for hepatitis C virus (HCV). And anemia is a major problem during therapy .Interferon and Ribavirin is the only line available now to stop this disease The aim of this work was to study the thyroid function in cases developed anemia during INF therapy. *Methods.* 371 Patients treated with combinations therapy for chronic hepatitis C between Sep. 2003 and March. 2009 were analyzed retrospectively. All patients were genotype 4 and received 48 weeks of standard doses of combined INF and Ribavirin. Anemia-related parameters were measured before and during treatment. Thyroid stimulating hormone (TSH) level was performed before initiating treatment and then every 3 months during the antiviral treatment. Thyroid dysfunction (TD) was defined as having hypothyroidism (TSH levels >4.0 mIU/L) or hyperthyroidism (TSH of <0.4 mU/L plus FT4 levels >24 and/or FT3 levels >5.5 pmol/L). *Results.* Thirty two (8.6%) patients developed TD during treatment, 27 (84%) of them were females, and the mean age was 41.03±10.2 years. Hypothyroidism (HYPO) developed in 26 cases (81.25%), 23 of them (88.5%) were females and hyperthyroidism was observed in 6 (18.75 %) patients, four of them (66.7 %) were females. In those with hypothyroidism, twenty (76.9%) patients were symptomatic and required L-thyroxin therapy. In those with hyperthyroidism (HYPER), all patients were symptomatic and required therapy. Anemia was almost universal during treatment. In HYPO Hb levels were 13.29±1.57 (10.6-16.4) , 11.11±1.62 (8.-14.3) , and 10.54±1.39 (8.50-13.6) gm/dl before, 3 months and 6 months after therapy respectively. In HYPER Hb levels were 13.73±1.66 (11.2- 16.5) , 11.62 ±0.89 (10.2-13.8) and 11.15±1.19 (9.1-13.5) gm/dl before, 3 months and 6 months after therapy respectively. *Conclusion.* thyroid dysfunction is a frequent complication during INF therapy and the occurrence of anemia is more frequent in this disorder. Hypofunction is more common than hyperfunction. Detection of the thyroid disorder is mandatory to treat anemia as a possible correctable factor to continue the interferon therapy.

1812

PLASMA LEVELS OF ADVANCED GLYCATION END-PRODUCTS ARE ASSOCIATED WITH HEMOLYSIS-RELATED ORGAN COMPLICATIONS IN SICKLE CELL PATIENTS

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Background. Oxidative stress plays an important role in the pathophysiology of sickle cell disease (SCD). Plasma levels of advanced glycation end-products (AGEs) are increased under oxidative conditions and are associated with disease severity in diabetes and inflammatory diseases. *Aims.* We questioned whether AGEs are increased in sickle cell patients and whether they are associated with SCD related complications. *Methods.* Plasma levels of the AGEs pentosidine, Nε-(carboxymethyl)lysine (CML) and Nε-(carboxy-ethyl)lysine (CEL)) were measured using single-column high performance liquid chromatography with fluorescence detection (pentosidine) and ultra performance liquid chromatography-tandem mass spectrometry (CML and CEL). *Results.* Plasma levels of pentosidine and CML were increased in HbSS/HbSβ0-thalassemia (n=60) and HbSC/HbSβ+-thalassemia (n=42) patients during steady state (P<0.01) as compared to healthy HbAA controls (n=30) without increments during painful crisis. CEL levels were comparable between all groups. Pentosidine and CML levels during the clinically asymptomatic state correlated significantly to hemolytic rate and were associated with the number of hemolysis-related organ complications (P<0.05). *Conclusions.* The increased plasma AGE levels in sickle cell patients and their association with hemolysis and hemolysis-related complications suggest that AGEs might be implicated in the pathophysiology of the hemolytic phenotype of SCD. Measurement of AGEs might be useful in predicting organ complications in SCD.

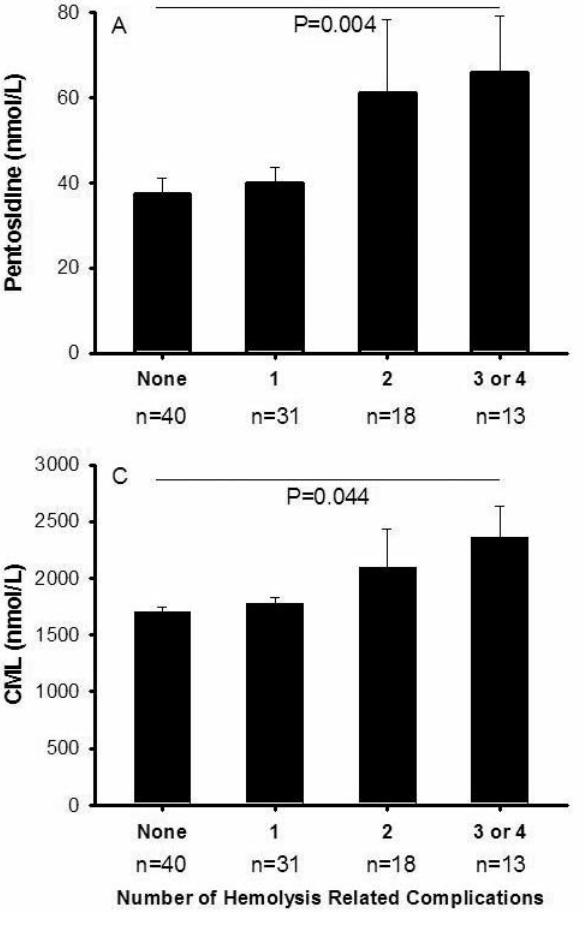

Figure. AGEs in relation to SCD related complications.

1813

EVALUATION OF GROWTH PARAMETERS AND PUBERTAL CONDITIONS IN PATIENTS WITH BETA-THALASSEMIA MAJOR

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Summary. Objective: multiple blood transfusions in Thalassemia Major patients causes iron overload in various tissues including endocrine glands thereby leading to multiple endocrine dysfunction. As the life expectancy of these children is increasing by transfusions and chelation therapy, endocrine complications are gaining more importance thus impairing the quality of life. The aim of this study was to determine the frequency of the growth retardation and the delayed puberty, possible causes of these complications and the effectiveness of the various chelation therapy given to the patients in our center. *Materials and methods.* Fifty-nine transfusion-dependent patients with Beta Thalassemia Major above six years of age followed up at the Istanbul Bakirkoy Maternity and Children's Research and Education Hospital were included in this study. All the information of the patients including age, gender, age of diagnosis, height, weight, parental height, target height, height velocity, puberty stage by Tanner method, treatments, volume of transfusions per month, mean ferritin levels and hemoglobin concentrations in the last two years, bone age, TSH, fT4, LH, FSH, estradiol or testosterone, cortisol, IGF, IGFBP-3 were recorded. Growth hormone and LH-RH stimulation tests were performed in selected patients. *Results.* 59 patients (33 female and 26 male), aged 13,40±4,66 (range 6,5-24 years) were studied. Mean age of diagnosis was 1,65±1,63 (0-6) years. 23 of our patients (%39) had at least one endocrinopathy. Of all patients, 16 (%27,1) had growth retardation, 8 (%13,5) had delayed puberty, 4 (%6,8) had hypothyroidism. Any of our patients did not have adrenal insufficiency. Growth hormone deficiency was determined at a rate of %15,3 in our patients. The mean ferritin level of patients was 2069±1306, the average hemoglobin level was 8.69±0.65. The mean heights of our patients were significantly less than their target heights. The patients with mean ferritin levels above 2500 ng/dL did significantly have short stature. There was a significant association between hypogonadism and short stature and also there was a significant association between the mean ferritin levels and the delayed puberty in our study. Growth retardation, height velocity, hypotiroidism and hypogonadism did not differ significantly amongst those having different chelating agents. *Conclusions.* Endocrine abnormalities should be monitored carefully and a thorough endocrine evaluation should be carried out yearly to detect endocrinopaties in every patient with thalassemia major. In conclusion, we thought that to keep the levels of ferritin down to the notable values by adequate and compatible chelation therapy will affect the growth and puberty conditions positively.

1814

CHARACTERIZATION OF A NEW HEMOGLOBIN VARIANT: HB SEVILLA BETA 81(EF5) LEU_PHE

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The haemoglobinopathies are the more frequent monogenic alterations, nevertheless theirs detection, unless there are clinical pathology, it is very difficult, since the majority these hemoglobin variant are due to mutations which only produce changes in the surface of the molecule and that do not show clinical manifestations. In this sense, we present a new structural variant of hemoglobin which detected during a control of glycoside hemoglobin. (HbA1c). At the propositus, a 61-year-old Spanish male, with diabetes mellitus type II, an anomalous hemoglobin having had been detected during a control of HbA1c by HPLC, and on not having coincided with any known hemoglobin. The new mutation β81(EF5) Leu→Phe, has been named Hb Sevilla after the city where the proband lives. Blood samples were collected with EDTA as anticoagulant and red cell indices were determinated on an automated blood cell counter, Hb 14g/dL; MCV 97.6fL; MCH 33.1pg; PCV 41.2%; RDW 13.4% and reticolocytes 1.2%. The HbA2 (2.4%) and HbF (0%) was measured by weak cation-exchange HPLC. In the same study an Hb X, which eluted before Hb A was isolated and measured (39.6%). Reverse phase HPLC study revealed the presence of an abnormal β-globin chain (β^X, β^A y α^A). Instability test by isopropanol demonstrated normality. Oxygen binding studies (Hemox Analyzer) showed normal whole blood oxygen affinity (P50 25 mmHg). For molecular characterization, genomic DNA from leukocytes of peripheral whole was automatic extracted with a Bio-Robot EZ1 (Quiagen). Selective amplification of the β gene was performed in an Applied Biosystems 2720 Thermal Cycler by PCR. Sequencing of the b gene in both directions, in an automatic sequencing (3.77 ABI, Applied Biosystems, CA), established a substitution of CTC→TTC at codon 81. This produces the Leu→Phe change at position 81 of the β-globin chain. Hb Sevilla is the third Hb variant resulting from Leu→Phe mutation at β81(EF5). In this case, neither the function nor to the stability of the hemoglobin involve modification, because the change is owing to substitution of an animo acid apolar hydrophobic (Leu) for other of the same characteristics (Phe), in the position 5 of the EF helix (such 3D position is 2,3-DPG binding site). The other two variants with substitution in the same position, Hb La Roche-sur-Yon (→His) and Hb Baylor (→Arg) are unstable and present an affinity for the oxygen increased, being these properties more accentuated in the Hb Baylor, probably due to the fact that the Arg possesses a guanidine group with electric charge, whereas the His has a imidazol group weakly ionized. Nevertheless in three cases, the substitution of the residue Leu in this position originates structural changes in the molecule that allow its detection by electrophoretic and chromatographic methods.

1815

SICKLE CELL DISEASE: A SINGLE CENTER REVIEW OF THROMBOEMBOLIC DISEASE IN A TERTIARY HOSPITAL IN RIYADH, SAUDI ARABIA

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Background/Aim. Sickle Cell Disease (SCD) is very common in Saudi Arabia and is considered as one of the major causes of morbidity. Pulmonary disease including thromboembolic disease (TED) accounts for the large portion of the morbidity of SCD. However, unclear evidences tried to implicate SCD as a risk factor for TED, thus this study was conducted to determine the prevalence and association of TED with SCD in our institution. *Methods.* Medical records of all patients diagnosed with SCD at the hematology clinic of King Khalid University Hospital, King Saud University, Riyadh, Saudi Arabia between 1982 and 2008 were retrospectively analyzed. Demographic characteristics, co-morbidities, SCD type, laboratory and coagulation profiles, treatment, mortality and cause of death were recorded. Occurrence of adverse events including stroke, DVT and infarction were also recorded. *Results.* A total of 478 patients with mean age of 21.3±11.1 years were included in the study, 51.5% were males. Avascular necrosis was documented in 39 (8.2%) patients, bone diseases in 21 (4.4%) patients and infections (both bacterial and viral) in 103 (21.5%) patients. Splenomegaly was documented in 176 (36.8%) patients wherein splenectomy was done in 19 (4.0%) patients. Mean D-dimer level was 442.3±933.5 mg/L, mean ferritin level was 1506±2570 ng/mL, mean hemoglobin was 9.1±1.7 g/dL, mean platelet count was 421.8±190.3×10^9/L, mean white blood cell count was 12.7±5.9×10^9/ and mean LDH was 386.4±223.3 U/L. Thromboembolic disease including deep vein thrombosis (DVT), pulmonary embolism (PE) and stroke was documented in 32 (6.7%) patients. Sixteen patients had stroke, 9 had DVT and 7 had pulmonary embolism. The occurrence of thromboembolic events was significantly associated with thromboembolism (P=0.017), age (P=0.018), D-dimer level (P<0.0001), ferritin level (P<0.0001), hemoglobin level (P<0.0001), platelet level (P<0.0001) and wbc count (P<0.0001). *Conclusion.* TED among Saudi patients with SCD showed 6.7% prevalence. SCD patients may have higher risk for TED if these patients have concomitant infections, with high D-dimer level, high ferritin level, low hemoglobin and low platelet counts.

1816

FLOW CYTOMETRIC OSMOTIC FRAGILITY (FC-OF) CAN REPLACE THE TRADITIONAL OSMOTIC FRAGILITY (OF) TEST? PRELIMINARY RESULTS

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Background. FC-OF has recently been proposed as a novel diagnostic

test for diagnosis of hereditary spherocytosis (HS). It is a simple, quantitative, and cost-effective method to determine the erythrocyte OF; its reliability seems to be similar to the traditional OF method. *Aims.* A) To assess the usefulness of FC-OF for the diagnosis of HS. B) To determine correlation between FC-OF and traditional OF *Results. Methods.* Blood samples from 19 normal controls and 15 anemic patients (HS 8, non-spherocytic anemias 7) were collected by venopuncture or digital puncture. The FC-OF test was performed according to the method described by Won and Suh, based on the observation that red cells suspended in isotonic normal saline undergo hemolysis when exposed to deionized water. The percentage of residual red cells is measured sequentially by flow cytometry in real-time before and after adding deionized water to the suspension. Traditional unincubated and incubated OF tests and FC-OF method were simultaneously performed in 10 normal controls, 5 HS patients and 3 patients with non-spherocytic anemias. Between-groups comparison was done using the Kruskal Wallis test. Correlation between the traditional OF and the FC-OF was analyzed. *Results.* HS patients had a significantly lower percentage of residual red cells than normal controls and non-spherocytic anemias (P:0.0012) (Table). Six HS samples (75%) showed results lower than the minimum value obtained in normal controls. Relationship between FC-OF and unincubated traditional OF showed an exponential fit; log of percentage of residual cells *vs.* OF showed significant correlation (r2: 0.842). *Conclusions.* FC-OF seems to be a reliable test for demonstrating increased osmotic fragility of red blood cells. Our results for normal controls and non-spherocytic anemias agree with those reported in the original description of the method; results for HS showed higher mean and wider data dispersion than those reported by Won and Suh. However, as both trials included a small number of samples, these results should be further validated through studies including large number of patients. The use of this test together with eosin-5'-maleimide flow cytometry and hypertonic cryohemolysis tests would allow diagnosing HS with capillary blood samples.

Table.

Group	% of Residual Red Cells			
	Median	Minimum	Maximum	Mean ± SD
Normal controls	63.4	23.9	94.8	58.63 ± 22.38
Hereditary Spherocytosis	8.7	4.0	81.0	19.42 ± 26.49
Non-spherocytic anemias	85.0	60.7	95.1	77.69 ± 14.04

1817

EFFICACY AND HEMATOPOIETIC EFFECT OF DEFERASIROX IN TRANSFUSION-DEPENDENT IRON OVERLOADED CHILDREN IN KOREAN SOCIETY OF PEDIATRIC HEMATOLOGY ONCOLOGY: CICL670AKR04T

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Background and objectives. Deferasirox (DFX) is a once-daily oral iron chelator for patients chronically iron overloaded by repeated red cell transfusion. This study evaluated the safety and efficacy of DFX in transfusion-dependent iron overloaded children with anemia including aplastic anemia. *Methods.* Fifty five patients were treated with once-daily DFX of 20-30 mg/kg for 52 weeks. Hematologic findings, serum ferritin (SF), amount of transfusion, and adverse events (AE) for safety were assessed every 4 weeks. *Results.* Forty three patients completed the study. Eight children withdrew due to skin rash and loss of follow up. In ITT analysis, forty patients were diagnosed with aplastic anemia and fifteen had other anemias requiring transfusion. Initial median SF was 2,553 (1003~13002) ng/mL, and time interval between primary diagnosis and enrolment was 6.2 (0.7~24.7) years. SF in aplastic anemia decreased significantly after 12 weeks of DFX administration, but showed no major change in other forms of transfusion-dependent anemia. Twenty eight patients experienced 58 cases of AE's including gastrointestinal symptoms, elevated liver enzymes, skin rash, and mild proteinuria. All AE's disappeared after interruption of DFX for about 2 weeks. In PP analysis, number of transfusions diminished significantly in all patients after DFX treatment (P=0.05), and there was a tendency for transfusion requirement to decrease in aplastic anemia patients (P=0.08), although such a trend was not observed in other anemia. *Conclusions.* DFX at daily doses of 20-30 mg/kg was well tolerated. The administration of DFX resulted in both effective iron chelation and decreased transfusion requirement in aplastic anemia patients. Dose modification should be considered in other transfusion-dependent anemia except aplastic anemia.

1818

COMBINED CHELATION THERAPY WITH DEFERASIROX AND DEFEROXAMINE IN TRANSFUSION-DEPENDENT PATIENTS WITH THALASSEMIA

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Background. Treatment of transfusional iron overload in patients with thalassemia has significantly improved with the use of deferoxamine (DFO), deferiprone (DFP) and deferasirox (DFX). These agents have been mainly used as monotherapy. Combining these drugs is a hopeful regimen, which may increase efficacy and reduce toxicity. So far, only combination of DFO and DFX has been extensively studied and it has been applied in clinical practice. *Aim.* In this report, we review the efficacy and the safety of combination treatment with DFO/DFX in patients with transfusion-dependent thalassemia. *Patients-Methods.* Thirteen patients (mean age 32.5, range 25.3-38.5 years) were treated with DFX (30-35 mg/kg/d) and DFO (35-40 mg/kg 2-5 days/week) for 12 months. The reason for initiation of combination treatment was failure to improve iron overload with previous intensification therapeutic regimen, namely DFO and deferiprone combination therapy and/or use of maximum allowed dose of oral chelating agents. Efficacy of treatment was evaluated by changes in hepatic and cardiac iron overload, as estimated by MRI (T2* sequence) and in ferritin levels. Safety parameters included transaminases, creatinine and cystatin C levels. *Results.* Mean, SD and range of the baseline evaluation showed: ferritin: 4592 ng/mL±1755 (1893-7800 ng/mL), liver iron concentration (LIC): 6.2±10 mg/g d.w. (7.1-42.5 mgFe/g d.w.), cardiac T2*: 29.2 ms (4-40ms), and left ventricular ejection fraction (LVEF): 64.4±6.2 ms (51-72.5%). Five patients had severe hepatic iron overload (LIC>15 mgFe/g d.w.) and two patients severe cardiac siderosis (T2*<10ms). After 1 year of treatment, LIC significantly improved (P=0.023) by a mean change of -3.4 mg/g d.w. (range 8.5 to -9 mgFe/g d.w.), with 9 out of the 13 patients showing any improvement of >20% from baseline LIC value. Ferritin decreased by a mean of 575±2016 ng/mL (P>0.05). There were no significant changes in cardiac T2* and LVEF. Nevertheless, improvement of LVEF to normal range was noted in the 2 patients with baseline LVEF<56%. Treatment was well tolerated and no serious adverse events were noted. Creatinine, cystatine C and transaminases levels did not change during the treatment. Compliance was variable, with many patients not receiving the recommended DFO doses. The main factors which affected efficacy, were compliance and degree of baseline hepatic iron load. *Conclusions.* This series of patients showed that combination treatment of DFO and DFX may improve iron overload without being associated with any significant toxicity. Further studies are required to verify these initial observations.

1819

HIGH PREVALENCE OF IRON DEFICIENCY IN PATIENTS WITH VARIOUS HEMATOLOGICAL AND MALIGNANT DISEASES: A SINGLE CENTER STUDY IN 1989 SEQUENTIAL PATIENTS

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Background. Iron is essential for the function of oxygen-binding molecules and plays a critical role in many metabolic pathways. Even with adequate storage iron, a restricted iron supply may result in functional iron deficiency with the potential for iron-restricted erythropoiesis and consequently anemia and impaired quality of life (QoL). This has been associated with prolonged intensive care stay, increased time in hospital and longer systemic inflammatory response syndrome, fatigue and exercise intolerance. Repletion of iron in patients with iron deficiency (ID) improves QoL and symptoms, including cognitive and exercise performance. Data on essential parameters of iron metabolism have not been reported in large number of unselected patients with cancer

as yet. *Aims.* To evaluate the prevalence of iron deficiency (ID), absolute iron deficiency (AID) and functional iron deficiency (FID) in a large cohort of patients having transferrin saturation (TSAT) and serum ferritin levels available with an additional focus on a sub-population of multiple myeloma (MM) patients. *Methods.* Data from 1989 patients (median age: 69 years, range: 8-105 years) presenting sequentially at the Center for Oncology and Hematology, Wilhelminenspital, Vienna between October 01, 2009 and January 26, 2010, have retrospectively been collected. Patients presented at different stages of their disease or even may not have had established diagnosis at the time of testing. In patients with multiple testing during this period only the first sample taken has been included. TSAT (available in all patients), serum ferritin (available in 1310 patients), serum iron, CRP, complete blood count, and other blood chemistry parameters have been determined using standard techniques. Commonly used definitions for ID (TSAT <20%), AID (TSAT <20% and serum ferritin <30 ng/mL) and for FID (TSAT <20% and serum ferritin ≥ 100 ng/mL) have been applied. *Results.* In the entire cohort of 1989 patients, iron deficiency was found in 870 (44%) and ID with anemia as defined by hemoglobin <12g/dL, in 420 (21%) patients. Cochran-Armitage trend test detected a trend for TSAT<20% across age groupings from 30-60 to >80 years (P<0.05). ID with anemia was found in 17% (103/612) of the patients 60 years of age or younger and increased significantly to 32% (118/365) in patients older than 80 years of age (P<0.0001). In the subgroup of 1310 patients with TSAT, serum ferritin and Hb available, ID was observed in 613 (47%), AID in 124 (9%), and AID with anemia in 72 (5%) of patients, respectively. FID was seen in 321 (25%) and FID with anemia in 176 (13%). For the myeloma subgroup a similar pattern was observed (Table 1). *Conclusions.* The results of this single center study show a high prevalence of ID and of FID in patients with various hematological and malignant diseases. The incidence of ID with and without anemia significantly increased with age. Inflammatory cytokines most likely account for the high ferritin values and the impaired availability of iron, diminished erythropoiesis and anemia. Whether parenteral iron supplementation will overcome the FID and improve the sequels of anemia in cancer patients should be investigated in prospective studies.

Table 1. Iron parameters and hemoglobin in patients studied.

	Sub group	TSAT<20% n (%)	TSAT<20% + Hb<12g/dL n (%)
All patients n=1989	**All, n=1989**	**870 (44%)**	**420 (21%)**
	Age <30, n=46 (2%)	26 (57%)	11 (24%)
	Age 30-60, n=566 (28%)	246 (43%)	92 (16%)
	Age 61-70, n=574 (29%)	212 (37%)	104 (18%)
	Age 71-80, n=438 (22%)	197 (45%)	95 (22%)
	Age >80, n=365 (18%)	189 (52%)	118 (32%)
TSAT and ferritin (ng/mL) available n=1310	**Any ferritin level**	**613 (47%)**	**323 (25%)**
	ferritin <30	124 (9%)	72 (5%)
	ferritin 30-100	168 (13%)	75 (6%)
	ferritin ≥100	321 (25%)	176 (13%)
	ferritin ≥800	45 (3%)	30 (2%)
Multiple Myeloma n=123	**Any ferritin level**	**44 (36%)**	**21 (17%)**
	ferritin <30	7 (6%)	2 (2%)
	ferritin 30-100	14 (11%)	8 (7%)
	ferritin ≥100	23 (19%)	11 (9%)
	ferritin ≥800	1 (1%)	1 (1%)

1820

TMPRSS6 GENE - TWO NEW NONSENSE MUTATIONS ASSOCIATED WITH IRIDA

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TMPRSS6 gene encodes a transmembrane serine protease expressed by the liver. *Iron.* Refractory Iron Deficiency Anemia (IRIDA) has shown to be caused by mutations in the gene TMPRSS6. IRIDA is an autossomal recessive disorder characterized by iron deficiency anemia unresponsive to oral iron treatment but partially responsive to parenteral iron therapy. IRIDA patients show high levels of hepcidin, a circulating hormone produced by the liver that inhibits both iron absortion from the intestine and iron release from macrophages stores. TMPRSS6 gene down regulate hepcidin expression by the liver. *Aim.* Report two cases of IRIDA with new mutations in TMPRSS6 gene. *Methods.* Molecular studies were performed in genomic DNA extracted from peripheral blood leucocytes and mutations in HBA, HBB and TMPRSS6 gene investigated by direct sequencing. *Results.* Case 1 - A 5 year-old boy with Hb - 7.9 g/dL, VGM - 53,9 fL, ferritin - 45,5 ng/mL, normal hemoglobin electrophoresis, non-invasive screening methods to gastrointestinal tract - negative, refractory to oral iron treatment. Sequencing of the alpha and beta globin genes was normal. Sequencing of the TMPRSS6 gene with a direct and a reverse primer revealed a CD603 GGG-AGG in homozigoty. Both parents presenting normal haematological parameters, are carriers of this mutation. Case 2 - A 5 year-old girl with microcitic anemia (Hb -10,3 g/dL, VGM -70,1 fL), ferritin - 19,6 ng/mL, normal hemoglobin electrophoresis, refractory to oral iron treatment. Sequencing of the alpha and beta globin genes was normal. Sequencing of the TMPRSS6 gene with a direct and a reverse primer revealed heterozigoty for a CD521 GAC-AAT mutation. No other mutation was found in the TMPRSS6. Her mother is also a carrier of the CD521 GAC-AAT mutation and her father is normal. *Conclusion.* We report two cases of non thalassemic hypochromic microcytic anemia, caused by inherited mutations in TMPRSS6 gene. These are two new nonsense mutations in TMPRSS6 gene not described on literature yet. In the first case the mutation, CD603 GGG-AGG, was found in homozygozity, in agreement with the fact that IRIDA due to TMPRSS6 mutations is a recessive disorder. In the second case only one mutation in the TMPRSS6 was found, not enough to justify the phenotype, further studies must be done to look for an association between this mutation and other genes mutations, like DMT1.

1821

A T2* MRI PROSPECTIVE SURVEY ON HEART AND LIVER IRON IN THALASSEMIA MAJOR PATIENTS TREATED WITH SEQUENTIAL DEFERIPRONE-DESFERRIOXAMINE VERSUS DEFERIPRON AND DESFERRIOXAMINE IN MONOTHERAPY

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Background. Most deaths in thalassemia major (TM) result from cardiac complications due to iron overload. No data are available in literature about possible different changes in cardiac and liver iron in TM patients treated with sequential deferipron-deferoxamine (DFP-DFO) *vs.* deferipron (DFP) and deferoxamine (DFO) in monotherapy. Magnetic Resonance (MR) is the unique non invasive suitable technique to evaluate quantitatively this issue. *Aims.* The aim of this multi-centre study was to assess prospectively in the clinical practice the efficacy of the DFP-DFO *vs.* DFP and DFO in monotherapy in a cohort of TM patients by quantitative MR. *Methods.* Among the first 739 TM patients enrolled in the MIOT (Myocardial Iron Overload in Thalassemia) network, 253 patients performed a MR follow up study at 18±3 months according to the protocol. We evaluated prospectively the 25 patients treated with DFP-DFO *vs.* the 30 patients treated with DFP and the 66 patients treated with DFO between the 2 MR scans. Myocardial and liver iron concentrations were measured by T2* multislice multiecho technique. *Results.* The doses of the sequential treatment were DFP 70±14 mg/kg/d for 4 d/w and DFO 42±8 mg/kg/d for 3 d/w, the dose of DFP was 73±16 mg/kg/d, DFO was 41±7 mg/kg/d for 5.5 d/w. Excellent/good levels of compliance were similar in the 3 groups (DFP-DFO 96% *vs.* DFP 97% *vs.* DFO 92%; P=0.67). Among the patients with no significant myocardial iron overload at baseline (global heart T2* ≥ 20 ms), there were no significant differences between groups to maintain the patients without myocardial iron overload (DFP-DFO 95% *vs.* DFP 100% *vs.* DFO 100%; P=0.23). Among the patients with myocardial iron overload at baseline (global heart T2* <20 ms), only DFP and DFO showed a significant improvement in the global heart T2* value (P=0.001 and P=0.003, respectively) and in the number of segment with a normal T2* value (P=0.031 and P=0.0001, respectively). The improvement in the global heart T2* was significantly different among groups (mean difference global heart T2* DFP-DFO 2.2±4.1 ms, DFP

10.7±7.2, DFO 3.6±5.4; P=0.007). The improvement in the global heart T2* was significantly lower in the DFP-DFO *vs.* DFP group (P=0.014), but it was not significantly different in the DFP-DFO *vs.* the DFO group (P=0.63) (see the Figure). In patients with liver iron overload at baseline (liver T2* <5.1 ms), the change in the liver T2* was not significantly different among groups (mean difference liver T2* DFP-DFO 0.9±2.1 ms, DFP 2.3±5.8, DFO 2.9±4.9; P=0.58). *Conclusions.* prospectively in a clinical setting over 15 months we did not find significant differences on cardiac and liver iron in TM patients treated with sequential DFP-DFO *vs.* the TM patients treated with DFO. Conversely, DFP monotherapy was significantly more effective than DFP-DFO in improving myocardial siderosis.

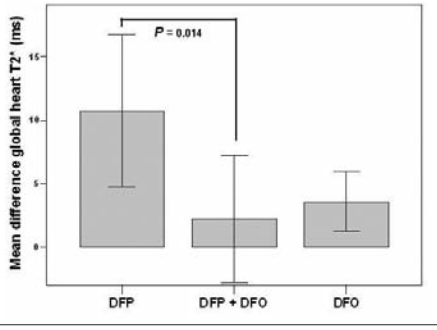

Figure.

1822

A T2* MRI PROSPECTIVE SURVEY ON HEART AND LIVER IRON IN THALASSEMIA MAJOR PATIENTS TREATED WITH SEQUENTIAL DEFERIPRON-DESFERRIOXAMINE VERSUS DEFERASIROX

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Background. Most deaths in thalassemia major (TM) result from cardiac complications due to iron overload. No data are available in literature about possible different changes in cardiac and liver iron in TM patients treated with sequential deferipron-deferoxamine (DFP-DFO) *vs.* deferasirox (DFX). Magnetic Resonance (MR) is the unique non invasive suitable technique to evaluated quantitatively this issue. *Aims.* The aim of this multi-centre study was to assess prospectively in the clinical practice the efficacy of the DFP-DFO *vs.* DFX in a cohort of TM patients by quantitative MR. *Methods.* Among the first 739 TM patients enrolled in the MIOT (Myocardial Iron Overload in Thalassemia) network, 253 patients performed a MR follow up study at 18±3 months according to the protocol. We evaluated prospectively the 25 patients treated with DFP-DFO *vs.* the 44 patients treated with DFX between the 2 MR scans. Myocardial and liver iron concentrations were measured by T2* multislice multiecho technique. *Results.* The doses of the sequential treatment were DFP 70±14 mg/kg/d for 4 d/w and DFO 42±8 mg/kg/d for 3 d/w, the dose of DFX was 26±6 mg/kg/d. Excellent/good levels of compliance were similar in the 2 groups (DFP-DFO 96% *vs.* DFX 100%; P=0.36). At baseline the 2 groups were homogeneous for cardiac and liver iron. Among the patients with no significant myocardial iron overload at baseline (global heart T2* ≥ 20 ms), there were no significant differences between groups to maintain the patients without myocardial iron overload (DFP-DFO 95% *vs.* DFX 96%; P=1.0). Among the patients with myocardial iron overload at baseline (global heart T2* <20 ms), only in the DFX group there was a significant improvement in the global heart T2* value (11±5 ms at baseline *vs.* 16±8 at 18±3 months, P=0.0001) and in the number of segment with a normal T2* value (P=0.003). The improvement in the global heart T2* was not significantly difference in the DFP-DFO *vs.* the DFX group (mean difference global heart T2* 2.2±4.1 ms *vs.* 4.6±4.8 P=0.2). The changes in the mean serum ferritin level were not significantly different between groups. In patients with liver iron overload at baseline (liver T2* <5.1 ms), the change in the liver T2* was not significant between groups (mean difference liver T2* 0.9±2.1 ms *vs.* 2.4±5.2; P=0.3). *Conclusions.* Prospectively in the clinical setting over 15 months we did not find significant differences on cardiac and liver iron by quantitative MRI in TM patients treated with sequential DFP-DFO *vs.* the TM patients treated with DFX.

1823

EFFICACY AND SAFETY OF DEFERASIROX IN PATIENTS WITH BASELINE LIVER IRON CONCENTRATION (LIC) <7 OR =7 MG FE/G DW: RESULTS OF EPIC LIVER MAGNETIC RESONANCE IMAGING (MRI) SUBSTUDY

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Background. Patients enrolled in previous deferasirox studies were heavily iron-overloaded, therefore data are limited in those with low iron burden receiving ≥20 mg/kg/day. In the overall EPIC study, although deferasirox efficacy was primarily monitored using serum ferritin (SF), a substudy explored the potential role of non-invasive LIC measurement by R2-MRI. This analysis evaluates deferasirox in less *vs.* more heavily iron-overloaded patients (baseline LIC <7 *vs.* ≥7 mg Fe/g dw). *Aims.* To evaluate the efficacy and safety of deferasirox for maintaining LIC in patients with baseline LIC <7 and for reducing LIC in patients with baseline LIC ≥7. *Methods.* Patients eligible for EPIC and enrolled at selected centres involved in the MRI substudy were invited to participate; separate informed consent forms were provided. Patients were transfusion-dependent, aged ≥2 years with SF ≥1000ng/mL, or <1000 with a history of multiple transfusions (>20 transfusions or >100 mL/kg RBCs), and MRI-confirmed LIC >2. Deferasirox starting dose was 10-30 mg/kg/day depending on blood transfusion frequency; appropriate dose adjustments (range 0-40) were performed every 3 months. Efficacy was based on LIC/SF changes after 1 year; safety assessments included adverse event (AE) and laboratory parameter monitoring. *Results.* 374 patients were included, 71 and 303 with baseline LIC <7 and ≥7, respectively; baseline characteristics were generally comparable. Most patients had β-thalassaemia (81.7% and 85.5%); in each underlying anaemia >70% had baseline LIC ≥7. Most patients were previously chelated (84.5% and 92.4%), primarily with deferoxamine (70.7%) or deferoxamine-deferiprone (28.4%). During the study, mean actual deferasirox doses were 20.7±5.4 and 27.1±7.1mg/kg/day in LIC <7 and ≥7 cohorts, respectively. In line with therapeutic goals, mean LIC was maintained around baseline in LIC <7 cohort, and decreased significantly in LIC ≥7 (Figure).

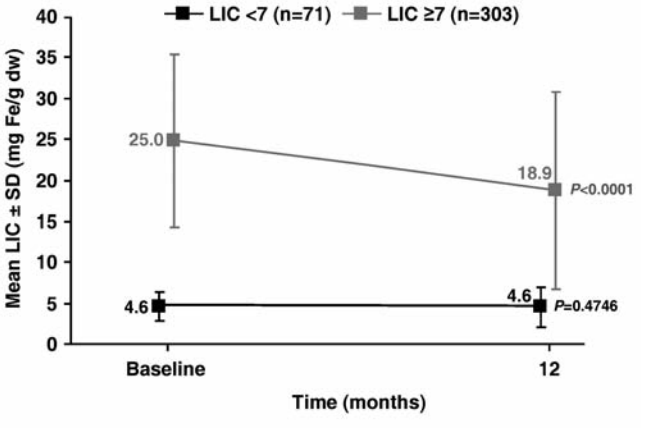

Figure. Absolute LIC during deferasirox treatment, by baseline LIC cohort.

Similar response was observed with median SF: 1479 to 1467 ng/mL in LIC <7 cohort (change -57), 4139 to 3176 ng/mL in LIC ≥7 cohort (change -830; P<0.0001). Sixty-three (88.7%) and 274 (90.4%) patients

completed 1 year. Three patients in LIC ≥7 cohort died (sepsis, n=2; pneumonia, n=1); none were considered drug-related. The most common investigator-assessed drug-related AEs were gastrointestinal, occurring more frequently in LIC <7 than ≥7 cohort (39.4% *vs.* 20.8%; P=0.001): diarrhoea (25.4% *vs.* 10.2%), abdominal pain (12.7% *vs.* 4.0%), upper abdominal pain (9.9% *vs.* 4.3%), constipation (9.9% *vs.* 3.0%). Most were mild/moderate and rarely led to dose decrease/discontinuation. Seven drug-related serious AEs were reported in LIC ≥7 cohort. Comparable proportions of patients had serum creatinine increases >33% above baseline and upper limit of normal (ULN; 7.0% *vs.* 5.3%) and alanine aminotransferase increases >10×ULN (1.4% *vs.* 0.7%). *Conclusions.* As per therapeutic objectives, a deferasirox dose of 20mg/kg/day maintained LIC/SF in patients with baseline LIC<7, whereas patients with LIC≥7 experienced significant reductions at a mean dose of 27mg/kg/day, confirming that iron balance is dose-related. Patients in LIC <7 cohort experienced more drug-related gastrointestinal AEs than LIC ≥7 cohort; neither age, underlying anaemia, nor deferasirox doses seemed to influence the differing frequencies, therefore it can be hypothesized that this may be related to lower iron burden in LIC <7 cohort.

1824

ANALYSIS OF TWO DIFFERENT SCHEMES OF IRON TREATMENT TO IMPROVE POSTOPERATIVE ANEMIA IN CARDIAC SURGERY. A RANDOMIZED, DOUBLE-DISGUISED, TRIPLE-BLIND STUDY

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Background. Anemia and increased transfusion requirements are common postoperative problems in cardiac surgery with cardiopulmonary bypass (CS-CPB). Several strategies have been designed and, although oral administration is the conventional route, intravenous (i.v.) iron has emerged as a safe and effective alternative for perioperative anemia management. *Aims.* To evaluate the efficacy and the impact of two different schemes of iron therapy on several clinical and analytical aspects in patients undergoing CS-CPB. *Methods.* We performed a prospective double-disguised triple-blind study in 159 patients (116 men, 43 women; mean age 65) undergoing elective CS-CPB. The patients were randomised in 3 groups: Group I (n=54) treated with i.v. iron sucrose, 100 mg/24 hours x3, pre- and postoperative and oral placebo (1 pill/24 h.) during the first month after discharge; Group II (n=53) receiving oral iron ferrous sulphate (80 mg/24 h.) pre- and postoperatively and up to one month after discharge, and an i.v. placebo while hospitalized; Group III (n=52) received an oral and i.v. placebo, both pre- and postoperatively. All groups were homogenous with respect to preoperative analytical data, demographic variables, comorbid conditions, surgical procedures and operative risk. Clinical and analytical variables were collected at different moments: preoperatively, operation room, Intensive Care Unit (ICU) admission and discharge, hospital discharge and one month after surgery. Comparisons were made to investigate if there was a difference in haemoglobin and hematocrit values, blood consumption, and median ICU and hospital length of stay. *Results.* We did not find significant differences between the haemoglobin and the hematocrit values at postoperative period or at 1 month after discharge between the 3 groups, but the highest values of serum ferritin and reticulocyte count were encountered in group I (P=0,002). Patients from group I also have: 1) A shorter postoperative stay (G-I 6.1 days, G-II 8.4, G-III 7.7), although the difference is not significant (P=0.169); 2) A shorter total hospitalization time (G-I 17.6 days, G-II 22.8, G-III 21.4) (P=0.07); 3) A slightly shorter ICU stay (G-I 3.7 days, G-II 4.5, G-III 5.3); 4) A decreased requirements of amines at ICU (G-I 65% did not need amines, G-II 43%, G-III 48%) (P=0.06); 5) A lower need of red blood cell transfusions (G-I mean 1.1 units, G-II 2.1, G-III 1.6). Globally, 63% of the patients in group I did not need blood transfusions (49% in group II and 50% in the placebo group). We also observed that the greater the consumption of blood the longer the stay at the ICU (P=0.001). Patients under 75 years in group I needed less transfusions at ICU when compared with placebo group (P=0,033). *Conclusions.* Intravenous iron achieves a significant elevation in ferritin and reticulocyte count at hospital discharge and 1 month after CS-CPB, but not in hemoglobin and hematocrit values, meaning that perhaps there is no clinical improvement in terms of correction of anemia. A trend toward shorter ICU stay, shorter postoperative stay, and shorter total time of hospitalization, is observed in patients treated with i.v. iron, as well as a lower consumption of blood transfusions.

1825

CELL-FREE DNA (CFDNA) AND INEFFECTIVE ERYTHROPOIESIS IN THALASSEMIA INTERMEDIA

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Background. Low concentrations (~ 30 ng or 5000 genome equivalents/mL) of cfDNA are found in the plasma of healthy individuals and increase in a number of conditions including cancer, chronic inflammation and trauma. The mechanisms of release of cfDNA in the blood are not well understood: DNA could originate from cells undergoing apoptosis/necrosis in tissues or from cells released in the blood and subsequently lysed. Also the tissue origin of cfDNA is unclear. It has been suggested that cfDNA, at least after bone-marrow transplantation, could be mostly hematopoietic in origin. This finding prompted us to explore whether cfDNA is increased in patients with ineffective erythropoiesis, a condition characterized by the increased proliferation and destruction of erythroid precursors, which is common in thalassemia. *Aim.* To study the behaviour of cfDNA in ineffective erythropoiesis caused by thalassemia and to assess whether cfDNA could be useful to evaluate the severity of ineffective erythropoiesis. *Methods.* We studied 51 thalassemia-intermedia patients (mean age 41 years, range 16-65), 24 of whom were splenectomized. On the basis of the genotype they were divided into three groups of increasing severity (Group 1: eterozygosis for β-thal.-ααα/αα; Group 2: genotype not included in Groups 1 and 3; Group 3: double eterozygosis or homozygosis for β+-defects). DNA was extracted with a chromatographic procedure from 200 μL of K2EDTA plasma and its concentration determined fluorometrically using the fluorescent dye PicoGreen that specifically binds to double-stranded DNA. In all patients the following parameters were also measured: hemoglobin, reticulocytes, peripheral erythroblasts, LDH, bilirubin, soluble transferrin receptor (sTfR). *Results.* In the 51 patients cfDNA ranged from 6 to 329 ng/mL with a significant difference of the distribution of values across the three groups (P=0,01). The highest mean concentration was observed in Group 3 (53+71 ng/mL) and the lowest in Group 1 (20+13 ng/mL). Comparing patients without and with splenectomy we observed a significant increase of cfDNA in splenectomized patients (22+12 *vs.* 49+63 ng/mL, P=0,001). In splenectomized patients cfDNA concentration was significantly correlated with erythroblasts (rho = 0,58 - P=0,003), LDH (rho = 0,60 - P=0,002), unconjugated bilirubin (rho = 0,52 - P=0,009). No correlation was observed between cfDNA and sTfR. *Conclusions.* Plasma cfDNA is increased in thalassemia intermedia in proportion with the severity of defects, but its concentration does not appear to correlate with the amount of ineffective erythropoiesis but only with the lysis of circulating erythroblasts which increases after splenectomy. It is likely that most erythroblasts undergoing apoptosis are engulfed by bone-marrow and spleen macrophages without releasing significant amounts of double-stranded DNA.

1826

ERYTHROCYTAPHERESIS IS NOT SUPERIOR TO WHOLE BLOOD PHLEBOTOMY IN PATIENTS WITH HEREDITARY HEMOCHROMATOSIS

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Background. Hereditary hemochromatosis (HH) may result in severe organ damage which can be prevented by therapy. Treatment with whole blood phlebotomy allows removal of excess iron and maintenance of low normal iron stores. The removed amount of iron may be increased two- or threefold for each procedure by using erythrocytapheresis, implying a reduced number of procedures and, possibly, more rapid achievement of normal iron stores and less use of time. Based on a couple of small, uncontrolled series and two small, randomized trials, some authors have concluded that apheresis is superior to phlebotomy. However, no larger, randomized study has been published. *Aim.* We wanted to study possible advantages and disadvantages of erythrocytapheresis as compared to phlebotomy in patients with HH. We report here the preliminary results. *Methods.*. In a prospective, randomized, open study performed from 2006 through 2009, patients with HH were randomized to bi-weekly apheresis or weekly whole blood phlebotomy. Prolongation of the treatment interval based on clinical assessment was allowed. Informed consent was obtained for all

patients. The minimum ferritin level required for inclusion was 300 microg/L for patients who were homozygous for the C282Y or H63D mutation, or compound heterozygous, while heterozygous individuals were eligible if ferritin was > 500 microg/L. Primary endpoints were decline of ferritin levels and transferrin saturation. Secondary endpoints were decline in hemoglobin levels, discomfort during the therapeutic procedure, and technician working time. *Results.* 67 patients were included, 9 women and 58 men, with a median transferrin saturation of 65% and median ferritin level 586 microg/L. 38 patients were homozygous for C282Y, 4 homozygous for H63D, 20 compound heterozygous and 5 heterozygous for C282Y. 33 patients were randomized to apheresis and 34 to phlebotomy. Ferritin levels < 100 microg/L were reached after a median number of 5 procedures in the apheresis group and 8 in the phlebotomy group, corresponding to 63 days in both groups. During this time, the median decline in ferritin levels was 497 microg/L (82% of baseline) in the apheresis group *vs.* 414 microg/L (74% of baseline) in the phlebotomy group; median decline in transferrin saturation, 30% (46% of baseline) *vs.* 33% (53% of baseline). Significant discomfort during or after the procedure was experienced at some time by 7 patients treated with apheresis and 6 of those treated with phlebotomy. None of these differences was statistically significant, and we observed no difference in decrease in hemoglobin levels. The mean sum of technician time consumption during the initial 63 day period was 286 minutes in the apheresis group *vs.* 238 in the phlebotomy group (P<0.001). *Conclusion.* We observed no significant differences in the rate of relative decline in ferritin levels, decline in transferring saturation, subjective discomfort or decrease in hemoglobin levels. The total technician time consumption was significantly higher in the apheresis group. In summary, we did not find any advantages of erythrocytapheresis as compared to conventional phlebotomy in the treatment of HH.

1827

FACTORS ASSOCIATED WITH IRON ACCUMULATION IN NON-TRANSFUSION-DEPENDENT THALASSAEMIA SYNDROMES: BASELINE DATA FROM MULTICENTER INTERNATIONAL DEFERASIROX STUDY (THALASSA)

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Background. Patients with genetically heterogeneous forms of thalassaemia, including β-thalassaemia intermedia (β-TI), HbE β-thalassaemia (HbE/β-T) and HbH disease, may not require transfusion therapy or receive only intermittent transfusions. However, recent studies have shown that iron overload still occurs due to increased gastrointestinal absorption of iron secondary to ineffective erythropoiesis, leading to the same clinical consequences as from repeated transfusions including liver, cardiac and endocrine dysfunction. Despite availability of effective iron chelation therapy (ICT), evidence suggests that monitoring and management of iron overload in non-transfusion-dependent thalassaemia (NTDT) patients is less rigorous than in thalassaemia major and other chronic anaemia patients and clinical studies are limited. A prospective, randomized, double-blind, placebo-controlled Phase II study, 'THALASSA,' evaluating the efficacy and safety of deferasirox in patients with NTDT is ongoing and will be the first large study evaluating ICT in these patients. *Aims.* To assess baseline characteristics, including iron parameters of NTDT patients from the THALASSA study. Additionally, a comparative analysis of baseline iron status and factors associated with iron accumulation in two predominating NTDT syndromes are presented. *Methods.* NTDT patients aged ≥10 years with liver iron concentration (LIC) ≥5 mg Fe/g dry weight (dw) [measured by R2 magnetic resonance imaging], and serum ferritin (SF) >300 ng/mL, are randomized 2:1/2:1 to starting doses of deferasirox 5 mg/kg/day:matching placebo/deferasirox 10 mg/kg/day:matching placebo over a planned 12-month period. Doses can be doubled after 6 months of treatment as required depending on LIC and change in LIC. Key exclusion criteria include: anticipated regular transfusions during the study (sporadic transfusions, as due to infection, permitted); any transfusion within 6 months or chelation therapy within 1 month prior to study start; HbS variants of thalassaemia; impaired renal and liver function. Primary efficacy endpoint is absolute change in LIC from baseline to 12 months. Safety assessments include adverse event and laboratory parameter monitoring. 156 patients are planned for inclusion. *Results.* As of January 2010, 92 patients have been randomized; baseline data are shown (Table). Forty-four (47.8%) patients were splenectomized; mean LIC was similar in splenectomized and non-splenectomized patients (16.8 *vs.* 15.2 mg Fe/g dw); although there were more splenectomized patients with LIC >15 mg Fe/g dw (47.7% *vs.* 39.6%). Mean SF was higher in splenectomized patients (1666 *vs.* 1032 ng/mL) and more splenectomized patients had SF >2500 ng/mL (15.9% *vs.* 2.1%). Patients with β-TI compared with HbE/β-T were older (mean 34.0 *vs.* 28.2 years), had received more blood transfusions prior to the study (median 9 *vs.* 6), and included more transfusion-naïve (20.0% *vs.* 2.8%) and splenectomized (75.0% *vs.* 33.3%) patients. Nevertheless, β-TI compared with HbE/β-T patients had lower SF (mean 1224 *vs.* 1546 ng/mL) and LIC (mean 14.6 *vs.* 17.5 mg Fe/g dw). *Conclusions.* Similar to transfusion-dependent thalassaemia patients, NTDT patients also develop iron overload shown by increased SF and LIC. The iron overload markers, SF and LIC were affected by splenectomy and underlying disease, respectively. This ongoing study will generate prospective efficacy and safety data for deferasirox in NTDT patients and may delineate pathophysiological mechanisms associated with iron overload.

Table.

Variables	Patients randomized, n=92
Disease, n (%)	
β-thalassaemia intermedia	40 (43.5)
α-thalassaemia disease	9 (9.8)
HbE/β-thalassaemia	36 (39.1)
Others	7 (7.6)
Age group (years), n (%)	
10–<16	8 (8.7)
16–50	77 (83.7)
>50	7 (7.6)
Male/female, n (%)	47 (51.1)/45 (48.9)
Race (Caucasian/Asian/Black/Other), n (%)	43 (46.7)/46 (50.0)/2 (2.2)/1 (1.1)
Any prior transfusion, n (%)	
Yes	81 (88.0)
No	11 (12.0)

1828

EVALUATION OF ANTIOXIDANT SYSTEMS IN HEALTHY CHILDREN RECEIVING PROPHYLACTIC IRON THERAPY

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Background. Iron is essential to many biochemical processes such as electron transfer reactions. It is recommended for supplementation of all children of certain ages in populations with a high prevalence of anemia. It has long been suspected that free radicals may play a role in iron induced cell toxicity because of the powerful pro-oxidant action of iron *in vitro*. Iron supplementation may result in the generation of free radicals and increase oxidative damage to DNA, proteins and lipids. *Aims.* The objective of this study was to investigate potential effects of iron supplementation at 4 month of age, for 2 months, on intraerythrocytic antioxidant enzymes. *Methods.* Four-month-old healthy infants who were born term and fed exclusively breast-milk were included in the study. None of the infants received iron supplementation previously and did not have either iron deficiency or iron deficiency anemia according to complete blood count and ferritin measurement. Twenty-six infants (14 male and 12 female) chosen randomly and not given iron supplementation constituted Group 1 (control group) and twenty-seven infants (16 male and 11 female) chosen randomly and given iron supplementation in the form of ferrous sulfate at a dose of 10 mg/d constituted Group 2. Weight, length, head circumference, complete blood count, serum ferritin level and intraerythrocytic zinc, iron, copper, catalase, malondialdehyde, superoxide dismutase and glutathione peroxidase levels were measured in the infants in both groups at 4 and 6 months of age. The study was approved by the local ethics committee. *Results.* There were no statistically significant differences for RBC, Hb, MCHC, Platelet, WBC and ferritin levels between the two groups at 4 month. After iron supplementation, RBC, Hb, RDW and ferritin levels of group 2 were higher than group 1 (P<0.05). Compared with control at 6 months of age no significant differences were observed for intraerythrocyte zinc, iron, copper, catalase, malondialdehyde, superoxide dismutase and glutathione peroxidase levels. *Conclusion.* Our study showed that the use of 10 mg/d of supplemental iron during 2 months in healthy iron-replete infants did not affect antioxidant status.

1829

EVALUATION OF UPPER AND LOWER RESPIRATORY FUNCTION IN PATIENTS WITH BETA-THALASSEMIA MAJOR

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Introduction. Life expectancy has greatly improved in patients with beta-thalassemia major (TM) due to regular transfusions and chelation therapy, however, complications still prevail. Hypoxia, hypercoagualability, iron overload and drug toxicity are factors attributing to multi-organ damage. Although impairment of lung function has been reported in thalassemic patients, possible involvement of the upper respiratory tract has not been evaluated. Aim. The aim of the study was to evaluate upper and as well as lower respiratory system function in patients with TM. *Patients and methods.* Fifty-two patients (25 males and 27 females), aged 9-34 years (mean age 21.3±6,2 years), were included in the study. All patients were receiving regular transfusions and chelation therapy. Patients with asthma, allergic rhinitis, pulmonary or cardiac disease and smokers were excluded from the study. Itn the study group mean pre-transfusion hemoglobin was 9,4±0,6 g/dL and mean serum feritin (as measured in the year prior of the study) was 1680.4±1404.1 ng/mL. All patients were measured for Peak Nasal Inspiratory Flow Rate (PNIFR) and Peak Inspiratory Flow Rate (PIFR) using Youlten rhinomanometer. The mucociliary clearance of the respiratory epithelium was measured using colour inert test (Edicol Orange 3% + CaHPO42H2O 97%). All patients underwent pulmonary function tests (PFTs) using an electronic spirometer. The diffusing capacity of the lungs was evaluated according to the value of the single breath carbon monoxide transfer factor (DLCO). This value was adjusted for the post-transfusional Hb (DLCO*). PNIFR, PIFR and the parameters of PFTs were measured and evaluated according to values matched for age, sex and anthropometric status. For PNIFR and PIFR a z-score was calculated. *Results.* Mean PNIFR was 175.56±33.8 l/min (z-score -1.56±1.21) and mean PIFR 258.3±43.95 l/min (z-score 0.59±1.06). Mean mucociliary clearance was 24.2±12.1min. In the study group 30/52 (57.7%) patients presented with normal PFTs, while 20/52 (38.46%) with restrictive pulmonary disease and a minority of 2/52 (3.84%) with obstructive disease. The diffusing capacity (DLCO* %) was decreased in 31/50 (62%) of the patients (mean=76.286 ±16.05). *Conclusions.* The study indicates that patients with TM do not demonstrate upper respiratory tract involvement. A restrictive type of pulmonary dysfunction and a decreased diffusing lung capacity are the predominant abnormalities, even in patients who in their majority are properly transfused and well-chelated.

1830

RETROSPECTIVE ANALYSIS OF IRON OVERLOAD IN PATIENTS WITH TRANSFUSION-DEPENDENT MYELODYSPLASTIC SYNDROME AND APLASTIC ANEMIA AND PROGNOSTIC VALUE OF IRON OVERLOAD STAGING SYSTEM

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Background. Recent retrospective studies showed iron overload is one of the poor prognostic factors in the transfusion-dependent patients of myelodysplastic syndrome (MDS) and aplastic anemia (AA). And recently transfusion-dependency was included as one of the independent prognostic factors of MDS in WPSS. However, iron overload is still to be elucidated especially in the context of organ damage, prognosis and treatment. *Aims.* Iron-mediated organ damage was analyzed in transfusion-dependent patients of MDS/AA and in addition prognostic value of iron overload staging system (Takatoku M *et al.*, Eur J Haematol; 2007) was verified in MDS patients. Methods. MDS/AA patients who had received over 40 units of red blood cell (RBC) during 2007 / 1 and 2009 / 12 were recruited from the thirteen related medical institutes of our district and were analyzed retrospectively. Iron overload staging system is the indication of the level of serum ferritin(SF), that is stage 1 (SF < 1000), stage 2 (2500 < SF > 1000), stage 3 (5000 < SF > 2500) and stage 4(SF > 5000). Results. Of the 167 registered patients 146 were eligible, MDS 96 (RA 64/RARS 2 / RAEB 20 /RAEB-T 2/CMML 2/OL 6), AA 34, MF 12, PRCA 4. The median sum of the transfused RBC was 98.5 units (40-646). 57/146 (39%) patients received iron chelation therapy (ICT). Of the 132 evaluated cases of iron-mediated organ damage, 120 (90.9%) were over 1000 ng/mL of SF level and 70 / 120 (53%) showed iron-mediated organ damage including of hepatic 55 (33.7%), renal 26 (17.8%), glucose metabolism 23 (15.8%), cardiac 20 (13.7%). Cases of iron-mediated organ damage (+/-) were (75/57) and SF were 4090±3099/2244±1498 (P= 0.01). Abnormal elevation of AST / ALT were more frequent in the iron overload stage 4 than in the stage 1 (P<0.05). Total mortality was 45 (30.8%), including infection 17 (37.8%), progression of primary disease 10 (22.2%), cardiac failure 8 (17.8%), but other iron-mediated organ damage were rather rare. Comparing to the iron overload stage 1, the stage 4 of RA / RARS implied poor prognosis, which was compatible to the previous report Malcovati L *et al.*, JCO, 2005) showing iron overload as possible poor prognostic factor in low grade MDS. Conclusions. Most patients of transfusion-dependent MDS/AA showed iron overload, elevation of SF and iron-mediated organ damage. However, iron-mediated organ damage did not deteriorate OS directly. Advanced stage of iron overload correlated with iron-mediated hepatic damage and poor prognosis of low grade MDS. Active ICT was expected to improve the poor prognosis of stage 4 of heavily iron-overloaded patients of RA/RARS. On the basis of this analysis, prospective study is expected to elucidate the detailed mechanism of prognostic effect of Iron overload, and achieve established strategy of ICT.

1831

CORRELATIONS OF SERUM FERRITIN (SF) AND LIVER IRON CONCENTRATION (LIC) BEFORE AND AFTER 1 YEAR OF DEFERASIROX TREATMENT

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Background. LIC measurement by R2 magnetic resonance imaging (MRI) is an established, non-invasive method for assessing iron overload and monitoring chelation therapy. However, serial SF measurement remains the most common clinical approach. While SF broadly correlates with body iron and LIC, this relationship may differ depending on the severity and distribution of iron overload and on the chelator; it is therefore important to understand the relationship between these markers for a particular chelation regimen. This relationship was evaluated before and after 1-year deferasirox treatment in a subgroup of patients enrolled in EPIC. *Aims.* To evaluate the relationship between LIC and SF in transfusion-dependent patients with iron overload. *Methods.* Transfusion-dependent patients aged ≥2 years with SF≥1000 ng/mL, or <1000 with a history of multiple transfusions (>20 transfusions or >100 mL/kg red blood cells) and MRI-confirmed LIC>2 mg Fe/g dw, were enrolled in EPIC. Deferasirox starting dose was 10-30 mg/kg/day depending on blood transfusion frequency; appropriate dose adjustments (range 0-40) were performed every 3 months. LIC (in mg Fe/g dw) and SF (in ng/mL) were assessed at baseline and after 1 year; correlations were evaluated based on the Pearson correlation coefficient. *Results.* Overall, mean LIC and median SF were significantly reduced by 5 and 555, respectively, (P<0.001). For patients with baseline SF<4000, LIC correlated significantly with SF at baseline (r=0.59, P<0.0001); the relationship was weaker for those with baseline SF≥4000 (r=0.19, P=0.02). A similar relationship was noted for patients with baseline LIC<20 (r=0.46, P<0.0001) or ≥20 (r=0.21, P=0.0053). For patients with baseline LIC≥20 and SF≥4000, there was no significant baseline correlation (r=0.06, P=0.54). However, after 1 year of deferasirox the relationship was restored in all high iron burden categories (SF≥4000, r=0.48; LIC≥20, r=0.55; LIC≥20 and SF≥4000, r=0.45;

P<0.0001 for all). Overall, there was a correlation shift so that a given SF (eg 2000) reflected lower LIC after 1 year than at baseline (10 *vs.* 12 for baseline SF<4000; 24.5 *vs.* 29 for SF of 5000 in patients with baseline SF≥4000). The correlation between changes in LIC/SF was strongest at lower baseline LICs (LIC<7, r=0.70, P<0.0001; LIC<20, r=0.50, P<0.001) and baseline SF<4000 (r=0.51, P<0.0001) than at higher baseline LICs (≥20: r=0.29, P=0.0001) or SF (≥4000: r=0.37, P<0.0001). *Conclusions.* Although absolute SF values above 4000 become less reliable for predicting the absolute LIC, SF trends can still be used to monitor LIC changes with deferasirox over wide iron-overload ranges. However, the LIC/SF relationship changes after 1 year of treatment. As shown in previous studies, with SF<4000 most of it is iron-free, deriving from macrophages in a manner proportional to iron stores; at SF>4000, increasing proportions are iron-rich, deriving from damaged hepatocytes. The current results are consistent with this and explain the restoration of LIC/SF correlation after 1 year in patients with initially high SF. The proportionally greater decrease in LIC relative to SF for patients with baseline SF<4000 is consistent with previously observed preferential iron removal by deferasirox from hepatocytes *vs.* macrophages. SF changes may not fully reflect LIC decrements under these conditions.

1832

EFFICACY, PATIENT SATISFACTION AND ADHERENCE TO TREATMENT WITH DEFERASIROX DOSES OF =30 MG/KG/DAY IN PREVIOUSLY CHELATED IRON-OVERLOADED PATIENTS WITH BETA-THALASSAEMIA MAJOR

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Background. Patients with β-thalassaemia major (TM) receiving regular blood transfusions frequently have serum ferritin (SF) levels >2500 ng/mL, indicative of high iron burden and potential negative clinical sequelae, despite availability of effective iron chelation therapy (ICT). The large, prospective EPIC trial of the oral iron chelator deferasirox recruited 854 patients with TM who previously received deferoxamine (DFO), deferiprone (DFP), or both. Despite a mean 10.8 years of chelation, median baseline SF was 3139 ng/mL. Previous studies have demonstrated that while deferasirox doses of 20 mg/kg/day maintain SF levels, doses ≥30 mg/kg/day are needed to significantly reduce SF in TM patients with high transfusional iron overload. *Aim.* To evaluate the efficacy of deferasirox ≥30 mg/kg/day in reducing SF in TM patients irrespective of prior ICT and to describe satisfaction with and adherence to higher doses of treatment. *Methods.* Patients with TM (≥2 years) and transfusional iron overload (SF levels ≥1000 ng/mL [or <1000 ng/mL but history of multiple transfusions] and liver iron concentration >2 mg Fe/g dry weight [measured by R2 MRI]) were enrolled. Deferasirox dose was initially based on transfusion requirements, with dose adjustments based on SF trends and safety markers. Patients that were previously chelated with DFO or DFP monotherapy, or both, and received mean actual deferasirox doses ≥30 mg/kg/day over 1 year were included in this analysis. Efficacy was assessed as change in SF at 1 year from baseline. Patients completed a Satisfaction with ICT (SICT) questionnaire to assess satisfaction and adherence. *Results.* 129 previously chelated TM patients received mean actual deferasirox doses ≥30 mg/kg/day over 1 year; 83 (mean age 19.5±8.2 years) received prior monotherapy and 46 (mean age 23.0±7.2 years) received prior DFO-DFP therapy. Mean duration of prior chelation therapy was 11.7±7.7 and 14.5±7.9 years in the prior monotherapy and DFO-DFP patients, respectively. During the study, mean transfusional iron intake was similar for both groups (monotherapy: 0.36±0.17 *vs.* DFO-DFP therapy: 0.34±0.10 mg/kg/day). In the prior monotherapy group (mean deferasirox dose 34.1±3.9 mg/kg/day), median SF decreased from 4885 ng/mL at baseline to 4282 ng/mL after 1 year (Figure a) resulting in a median decrease of 1024 ng/mL (P<0.0001; last-observation-carried-forward [LOCF]). In the prior DFO-DFP group (mean deferasirox dose 33.9±2.2 mg/kg/day), median SF decreased from 5921 to 4327 ng/mL resulting in a median decrease of 886 ng/mL (P=0.0078; LOCF). Satisfaction and adherence data were available from 26 patients. Patients reported increased satisfaction with deferasirox in SICT domains for side effects, acceptance and burden of ICT; a trend in increased satisfaction with perceived effectiveness was noted. Patients also indicated improved persistence and adherence (Figure b). Five patients (3.9%) discontinued therapy, due to cardiac failure, increased transaminases, consent withdrawal, lost to follow-up and protocol violation (all n=1). Most common investigator-assessed drug-related AEs were rash (n=14, 10.9%) and diarrhoea (n=12, 9.3%). *Conclusions.* Deferasirox doses ≥30 mg/kg/day for 1 year led to significant and clinically relevant reductions in SF in these heavily transfused TM patients, irrespective of prior ICT with monotherapy or DFO-DFP therapy. Patient-reported satisfaction and adherence to treatment also improved.

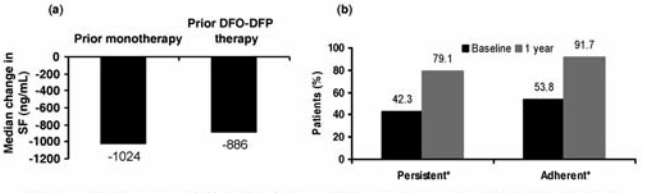

Figure. (a) Median change in SF (ng/mL) over 1 year and (b) patient-reported persistence and adherence in patients who had prior monotherapy or prior DFO-DFP therapy

1833

THE TREATMENT OF REFRACTORY FORM AUTOIMMUNE HEMOLYTIC ANEMIA BY RITUXIMAB

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AIHA in 30% are resistant for traditional kind of treatment. We observed 13 patients who had resistant form of AIHA with warm autoantibody and positive direct Coombs test and 2 patients with Evans syndrome. There were 7 men and 8 women, range 19-60 y.o, had from 4 month up to 10 years of previous medical history. All patients were resistant for all kind of therapy: splenectomy for 11 patients, prednisolone, cyclophosphamide, azatioprine. The hemoglobin level during hemolyses reached 45 g/L (35-65 g/L), erythrocyte count was less $1,5\times10^{12}$, reticulocytes were in the range of 3,2% up to 34% , bilirubin level increased up to 2 - 4 times above normal. The therapy began from the high doze of methylprednisolone (500 mg, 3 days). After that Rituximab administered intravenously at a dose 375 mg/m^2, from 2 to 4 consecutive weeks. The patients received Rituximab during 2 consecutive weeks in case if B lymphocytes disappeared from the blood, at the 7 day at the beginning of the treatment. 13 patients achieved complete respond, duration 4-56 month. The first effect was seen in the period from 3 weeks - 2 month (mediana 1,5 month). 4 patients had responded in the period from 2,5 to 4 month. Controlling hemoglobin, reticulocytes, bilirubin and other parameters of hemolytic activity we could prevent the acute hemolytic crises for 7 patients, beginning the next courses of Rituximab, after 4-18 month remission. 5 patients had two courses of Rituximab, 2 patients - 4 courses. All patients had normal immunoglobulin (IgG, IgM) levels after Rituximab. Nobody had any bacterial or viral infections during or after treatment. 2 patients with viral hepatitis C and B hadn't any hepatitis activity after the treatment. *Conclusion.* The Rituximab is more effective in the treatment resistant form AIHA. The patients achieved long time remission. Between courses they didn't received any supportive therapy. Rituximab may be become alternative splenectomy for old patients.

1834

EVALUATION OF 'SILENT STROKE' IN PATIENTS WITH SICKLE CELL DISEASE BY MEASURING PLASMA LEVELS OF THE NEUROPROTEINS NSE, S100A1B-S100BB AND NT-PRO-BNP

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Background and aims. Patients with sickle cell disease (SCD), a chronic hemolytic anemia, present with a wide variety of neurological syndromes, including ischemic and hemorrhagic stroke, transient ischemic attacks, 'soft neurological signs', seizures, headache, coma, visual loss, altered mental status, cognitive difficulties, and covert or 'silent' infarction. The S100b and neuron-specific enolase (NSE) are proteins generally considered as markers of central nervous system(CNS) injury, measured both in plasma and/or CSF (cerebrospinal fluid). S100b protein is a calcium-binding protein in the cytoplasm of CNS astroglial and Schwann cells and indicates astrocytal damage as well as dysfunction of the blood-brain barrier (BBB). Neuron-specific enolase is an enzyme localized in neuronal axons and cytoplasm and plays a crucial role in glycolysis and is regarded as a marker for neuronal cell loss. In this study, we evaluated the "silent stroke"in adult patients with SCD at steady phase, by measuring the plasma levels of s100b, NSE and the neurohormone brain-natriuretic-peptide (BNP). *Patients and methods.* Twenty-six patients with SCD, aged 40.5±13.2 y mainly with mild and moderate severity type of the disease were included in the study, while 21 apparently healthy individuals of same age served as controls. Measurements of the plasma levels of the proteins, S100 (as heterodimer S100A1B and homodimer S100BB), NSE and NT-pro-BNP were performed using fully automated electrochemiluminescence assays on the immunochemistry autoanalyzer Roche cobas e411. *Results.* The main results of the study showed that: a) NSE levels were significantly higher in patients with SCD compared to those of controls 8.3±3.6 ng/mL *vs.* 2.3±1.4 ng/mL, P<0.004; b) S100 proteins concentrations were significantly lower in patients with SCD compared to those of controls 0.05±0.02 μg/L *vs.* 0.07±0.03 μg/L, P<0.02; c) NSE levels and NT-pro-BNP correlated positively (binomial-bidirectional regression, P<0.001, while d) no correlation was found between S100 proteins concentrations and NSE and/or NT-pro-BNP levels. *Conclusions.* The increased NSE levels indicate that "silent stroke"in patients with SCD is accompanied with neuronal cell loss. There is no clear explanation for the lower than normal S100 proteins level found in patients with SCD. We postulate that it is probably due to the chronic neurological impairment of SCD patients, as decrease of their level is mentioned after acute neurological events. Moreover, it seems that in low concentration of S100 proteins act as growth factor and induce proliferation and differentiation of the neurons, while high concentrations are toxic. With regard to the results of the present study, further research is necessary to evaluate the role of S100 and NSE in patients with SCD.

1835

THYROID HYPOFUNCTION A POSSIBLE IMPORTANT ETIOLOGY OF ANEMIA DURING INTERFERON THERAPY IN VIRUS C HEPATITIS

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The effect of interferon therapy on the development of the occurrence of anemia during virus c hepatitis therapy is always contributed to either the interferon effect on the immunomodulatory system leading to pernicious anemia. Or to the known effect of the concomitant Ribavirin leading to hemolytic anemia. Thyroid dysfunction was considered, but with a lesser important effect. The aim of this work was to study the thyroid hypofunction in cases developed anemia during the therapy and try to treat the dysfunction and detect its effect on the anemic state. In an attempt to find a solution to avoid therapy cessation as it is the only way to deal with disease. *Material and methods.* 70 anemic patients occurred during the treatment of standard doses of combined interferon and Ribavirin due to virus c hepatitis (Hb levels below 11 gm/dL). Hb and other anemic parameters, TSH and FT4 levels were measured. Those cases with TSH levels more than 4.5 mIU/L and normal or low FT4, they received levothyroxine therapy with a low dose then increasing until the daily dose of 100 microgram. Then TSH and FT4 levels were measured after 6 months. Correction of the anemic state considered if Hb is above 11gm/dL. *Results.* 70 patients (37 females and 33 males) had HB levels below 11 gm/dl were randomly selected. 37 patients had TSH levels more than 4.5 mIU/L and normal or low FT4 (53%). After levothyroxine intake and at the end of interferon therapy 29 patients out of 37 (78%) had Hb more than 11gm (P<0.05). *Conclusion.* during standard interferon therapy as the occurrence of anemia is a common finding, thyroid hypofunction is a common cause and replacement therapy is a good tool to deal with this complication and so the cessation of therapy may be avoided in most cases.

1836

THE EFFECT OF MATERNAL ANEMIA (IRON DEFICIENCY ANEMIA) ON PREGNANCY OUTCOME

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Background. Iron deficiency anemia (IDA) is relatively common in the third trimester of pregnancy, but causal associations with low birth weight, premature delivery, and low Apgar score are still under study. *Objective.* The aim of the study was to assess the relationship between maternal hemoglobin (IDA) and prenatal outcome (birth weight, gestational age, and Apgar score) in a cohort of 200 pregnant women in the third trimester and to highlight the importance of antenatal care regarding maternal health and fetal outcome. *Methods.* This study was performed on a cohort of 200 selected pregnant women in their third trimester that referred to Elzahra Hospital in Eljfara area for antenatal care and delivery during November 2008 till May 2009. The data collected were based on questionnaires, clinical examination and laboratory investigations. Hematologic and iron-status measures, pregnancy outcomes, and fetal and neonatal evaluations were compared between iron deficiency anemia pregnant women (n=66) and controls (n=132). *Results.* Complete data were available for the 200 women. Anemia (hemoglobin <11 g/dL) was present in 68 (34%) pregnant women, and iron deficiency anemia (Hb <11 g/dL & ferritin ≤9 ng/mL was present in 66 (97.1%) pregnant women out of the 68 anemic women. Also the results of our study showed no association between IDA and pregnancy outcome in the third trimester of pregnancy. *Conclusion.* Maternal anemia detected during the later stages of pregnancy, especially the third trimester, often reflects the expected (and necessary) expansion of maternal plasma volume. This might explain our findings in this study which showed no significant association between maternal iron deficiency anemia in the third trimester and the health of the infant at birth in terms of premature delivery (P 0.134), low body weight (P 0.082), and low Apgar score at 1 and 5 minutes (no association).

1837

APPROPRIATE EVALUATION OF TRANSFUSIONAL CARDIAC IRON OVERLOAD BY T2* AND R2* MAGNETIC RESONANCE

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Background. Cardiac dysfunction due to transfusional iron overload is one of the most harmful complications for patients with bone marrow failure syndromes. Magnetic resonance imaging (MRI) is known to correlate to tissue iron concentration. However, it has some disadvantages including increased cost of the examination and the relatively limited availability of MRI systems. On the other hand, clinical parameters such as total red blood cell (RBC) transfusion units and serum ferritin levels are usually considered as indicators for iron chelation therapy. *Aims.* To evaluate cardiac iron overload with MRI-T2* and MRI-R2* (reciprocal values of T2*) values, and to find out whether these values corroborate some known clinical parameters based on retrospective data in blood transfusion-dependent patients. *Methods.* We examined MRI-T2*, -R2* values of myocardium and left ventricular ejection fraction (LVEF) by ultrasonography in 17 adult patients (12 men and 5 women) in our institution with blood transfusion-dependent bone marrow failure syndromes. Patients treated with anticancer drugs were excluded, since they can cause organ dysfunction. The 17 cases consisted of 6 patients with myelodysplastic syndrome, 7 with aplastic anemia and 4 with myelofibrosis. Their ages ranged from 35 to 88 (median 67 years) and none of them had previously received oral iron chelation therapy, while 9 of them had received intermittent intravenous deferoxamine. The data were analyzed to see the relationship with known parameters, such as total RBC transfusion units and serum ferritin levels. *Results.* We found a positive correlation in all patients between R2* values and serum fer-

ritin levels (r=0.83), and also the former and total RBC transfusion volume (r=0.93). The T2* values are more sensitive at the early stage of iron overload. In contrast, R2* values are more useful in the higher range. We also found that elevation of R2* values correlates with deterioration of LVEF (r=-0.72). From the formulae of R2* values, we concluded that approximately 60 Japanese RBC transfusion units or 2,000 ng/mL serum ferritin level might be the cutoff value indicating possible future cardiac dysfunction. *Summary/Conclusions.* We examined MRI-T2*, -R2* values of myocardium in 17 blood transfusion-dependent patients to find out the relationship between these values and known clinical parameters of iron overload such as serum ferritin level and total RBC transfusion units. As a result, R2* values correlated with serum ferritin levels, total RBC transfusion units and also with deterioration of LVEF. The results of our study and the formulae of R2* values which is made based on the results were compatible with the Japanese and international guidelines for iron chelation. However, there is a risk of overlooking the early stage of myocardial iron overload due to the heterogeneity of the disease. According to the fact that R2* values gradually rise as myocardial iron accumulates, it is recommended to consider the possibility of myocardial iron overload even when T2* values are in normal range and LVEF seems to be preserved.

1838

EFFICACY AND SAFETY OF DEFERASIROX (EXJADE_) IN PATIENTS WITH TRANSFUSION- DEPENDENT ANEMIAS: PRELIMINARY RESULTS FROM THE FIRST, RETROSPECTIVE, MULTICENTER BRAZILIAN STUDY

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Background. Deferasirox is a once-daily oral iron chelator with established dose-dependent efficacy for treating transfusional iron overload. *Aims.* to evaluate the efficacy and safety of fixed starting doses of deferasirox based on transfusion history, with subsequent dose titration based on serum ferritin (SF) trends in transfusion-dependent anemia patients. *Methods.* Pts had transfusion-dependent anemia with a history of multiple transfusions (>20 transfusions) and/or SF levels ≥ 1000 ng/mL and serum creatinine level < the upper limit of normal (ULN). Deferasirox starting dose was 10-30mg/kg/day depending on transfusion requirements and subsequent dose adjustments of 5-10 mg/Kg/day (range 0-35 mg/kg/d) were done every 3 months based on changes in SF and safety parameters. Efficacy was assessed monthly by measuring change from baseline in SF levels. Safety was evaluated on a monthly basis according to the incidence and type of adverse events and measurement of laboratory parameters, including serum creatinine and liver enzyme levels. *Results.* 105 pts (40 M, 65 F; mean age 25.0±16.6 yrs) were enrolled; 46% (n=48) aged <20 yrs; 54% Afro-descendant (n=57). Underlying anemias were: sickle cell disease (n=59), β-thalassemia (n=32), myelodysplastic syndromes (n=6) and other conditions associated with anemia (n=8). Most pts (79%, n=83) had received > 40 units of red blood cell (RBC); 71.5% (n=75) were on regular RBC transfusion. Sixty-four (61%) pts started on 20 mg/kg/d and 41(39%) > 20-30 mg/kg/d, 15.2% of pts had dose increases at a median of 24 weeks after treatment initiation. Mean±SD SF levels (μg/L) did significantly reduce at 6 months and 12 months compared to baseline (BL) [from 3132.14±2237.47 to 2784.25±1969.7 at 6 months (P=0.0001) and 2327.46±1873.8 at 12 months (P=0.005)]. The proportion of patients with SF levels < 2000, 2000-3000 and > 3000 μg/L from BL to 6 and 12 months by percentage of patients changed from 36% to 47.5% and 52%; from 26% to 26.5% and 24.5%; from 38% to 26% and 23%, respectively. No patient discontinued the treatment. No death was reported by the investigators during the study. The most common drug-related (investigator-assessed) AEs were mild, transient diarrhea (n=15; 14.3%), rash (n=5; 4.7%), nausea (n=9; 8.5%) and headache (n=6; 5.7%). Seven pts (6.6%) had serum creatinine value >33% above BL on two consecutive visits, 3 (2.8%) of whom had creatinine increases above the ULN; there were no progressive increases or renal failure. Eleven (10.5%) pts had an increase in alanine aminotransferase < 5× ULN but no one experience increases ≥ 5x ULN; levels were already elevated in all of them. *Conclusions.* This first multicenter Brazilian study confirms deferasirox efficacy in achieving a reduction of iron load across a wide range of pts with transfusion-related iron overload. Deferasirox was generally well tolerated in pediatric and adult pts with a safety profile consistent with data from previous clinical trials. The availability of deferasirox as a once-daily oral iron chelator would potentially facilitate improved compliance, and thereby reduce morbidity and mortality from iron overload.

1839

IRON DISTRIBUTION BETWEEN LIVER, SPLEEN, PANCREAS, BONE MARROW, AND MYOCARDIUM IN β-THALASSEMIA MAJOR: AN R2-MRI STUDY

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Background. Despite extensive research on MRI of hepatic and cardiac iron overload, there have been limited data on quantification of iron in organs other than liver and heart. *Aims.* a) to investigate the correlation between the degree of hepatic, splenic, pancreatic, vertebral bone marrow (VBM), and myocardial siderosis, as expressed by relaxation rate (R2 = 1/T2) values, b) to describe atypical MR imaging appearances of pancreas and spleen in transfusion-dependent patients with β-thalassemia major. *Methods.* From the archieves of our department, we retrieved the abdominal and cardiac MRI studies of 89 consecutive patients with β-thalassemia major (46 men and 43 women, aged 9-40 years, mean 28.2+7.5) which were performed in a 8-year period at the same 1.5 Tesla MR imager , for evaluation of liver and myocardial iron overload. The study protocol was approved by the board for retrospective studies of our institution. The MR imaging protocol included a single-slice respiratory triggered 16-echo Carr-Purcell-Meiboom-Gill (CPMG) spin echo sequence and subsequent calculation of R2 values of liver, spleen and VBM in all patients. Pancreatic R2 values were possible to determine in 58/89 patients. Additional multislice T1 axial - and T2 coronal sequences were applied. Imaging of the heart was performed in 81/89 patients, using a double oblique imaging plane while cardiac R2 was estimated at the left ventricular free wall. The same MRI protocol was applied in ten healthy controls, after informed consent. From patients' medical records, serum ferritin values closest to the MR exam (1-4 weeks) were retrieved. Statistical significance was set at P<0.05. *Results.* Hepatic R2 values were significantly increased in 85/89 patients; VBM, pancreatic, and myocardial R2 values were increased in 82/89, 36/58 and 55/81 patients, respectively. Decreased pancreatic R2 values combined with T1 hyperintensity were seen in 10 patients with severe hepatic siderosis and was attributed to fatty degeneration. Of the 44 nonsplenectomized patients, splenic R2 values were decreased in 35 and normal in nine patients; all the last ones exhibited splenomegaly. Hepatic R2 values correlated with splenic (r=0,589, P=0.001), VBM (r =0,405, p =0.002),and myocardial (r=0,357, P=0,0016). Serum ferritin levels were found to correlate with hepatic (r=0.73, P< 0.0001), bone marrow (r=0.39, P< 0.002), splenic (r=0.69, P<0.0001) R2 values and myocardial R2 (r= 0,39, P<0.01) but not with pancreatic R2 values. Pancreatic R2 values were not correlated with hepatic R2 ones or serum ferritin. *Conclusions.* Hepatic siderosis correlated with splenic, VBM and myocardial siderosis, as expressed by respective R2 values, although unpredictable patterns of iron distribution were seen. Normal splenic R2 values combined with splenomegaly were occasionally seen, that might be attributed to splenic dysfunction. Fatty degeneration of the pancreas was not uncommon; pancreatic R2 values can not be considered representative of iron deposition since the effect or iron interferes with that of fat for R2 calculations.

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TRANSIENT ELASTOGRAPHY (FIBROSCAN): A NEW AND SAFE TOOL FOR THE NONINVASIVE ESTIMATION OF LIVER FIBROSIS IN HEREDITARY HEMOGLOBINOPATHIES

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Background. Hepatic iron overload and chronic hepatitis are serious complications of chronic transfusion therapy in patients with hemoglobinopathies and play an important role in the development of hepatic fibrosis and cirrhosis. Transient elastography (Fibroscan, Echosens, Paris, France) is a new, noninvasive, rapid, reproducible, bedside method allowing the assessment of liver fibrosis by measuring the liver rigidity (Liver Stiffness Measurement, LSM, kPascals). *Aims.* To evaluate liver fibrosis with Fibroscan in patients with hemoglobinopathies and correlate the LSM with demographic characteristics, ALT, ferritin, transfusion frequency and iron chelating therapy. *Methods.* We evaluated 109 adult patients with hemoglobinopathies (48M/61F, mean age 46±9,5 years). Nineteen patients (17,4%) had Thalassemia Major (TM), twenty four (22%) had Thalassemia Intermedia (TI) and sixty six had Sickle Cell Disease (60,6%). All patients were evaluated for liver fibrosis with Fibroscan. Total iron burden was monitored by measuring serum ferritin levels , ALT and indices of chronic hepatitis B or C were monitored by conventional methods. All measurements were synchronous with Fibroscan evaluation. *Results.* The median LSM was 6 kPascals with a minimum value of 3 kPascals and a maximum value of 48 kPascals, (range 4-9). The median ALT was 24 IU/L (range 16-40), median ferritin was 374 ng/mL (range 148-642). Nineteen patients (20,2%) had indications of chronic hepatitis B or C. The three subgroups of patients according to subjecting hemoglobinopathy differed significantly in age (P=0,016), ALT (P=0,032) and ferritin levels (P=0,012) but there was no statistical significant difference in LSM (P=0,482). LSM was significantly correlated with ALT levels (r=0,565, P<0,001) and the presence of chronic hepatitis (r=0,419, P<0,001) while it was not correlated with ferritin levels (r=0,63, P=0,561). *Summary/Conclusions.* Fibroscan may constitute a reliable and easy to apply noninvasive means of detecting liver fibrosis in patients with hemoglobinopathies. It seems to correlate with the presence of chronic hepatic injury, reflected by increased ALT levels and chronic infection with hepatitis viruses. The finding that ferritin levels did not correlate with LSM was expected as ferritin is not a reliable index of liver iron concentration and iron induced liver injury. The absence of differences in LSM between subgroups could be explained by the small number of patients in each subgroup.

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A PROSPECTIVE OBSERVATIONAL REVIEW OF FERRIC CARBOXYMALTOSE ALONE IN THE IMPROVEMENT OF ANEMIA SECONDARY TO IRON DEFICIENCY IN CANCER PATIENTS

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Background. Iron is vital for all metabolic processes in the body: among others, iron-containing enzymes are essential for electron transport in the respiratory chain, transmission of nerve impulse, erythropoiesis, oxygen transport and supply to the brain and other tissues. Approximately 500 mg iron per liter of blood are required for normal hemoglobin levels. Restricted iron supply may lead to iron deficiency (ID) and, if this condition persists, to iron deficiency anemia (IDA). ID has been associated with reduced functional capacity and/or patient reported poor physical condition. These symptoms, even more manifest in the presence of anemia, may significantly decrease patient quality of life (QoL) and hence intervention is required. Within oncology, IV iron in conjunction with erythropoiesis stimulating agent (ESA) has been demonstrated to improve hemoglobin (Hb) levels. This response may originate from improved iron availability that overcomes iron restricted erythropoiesis due to increased iron demands from ESA use, as well as ID correction alone. *Aim.* Provide an initial observation of the role of intravenous (IV) iron alone in correction of anemia secondary to iron deficiency in cancer patients. *Methods.* A non-interventional observational study was initiated in December 2008 across 39 sites in Germany and is ongoing. To date, over 300 patients have consented with 163 having completed a 12-week observational period at time of analysis. Of these completed patients, 135 had no ESA use within 4 weeks of screening or during the observational period and received at least 1 dose of ferric carboxymaltose (FCM). Here we present an interim review of the aforementioned subset of patients focusing on the correction of anemia secondary to iron deficiency. *Results.* 135 patients constituting a cross section of tumors including colorectal (22%), breast (21%), and stomach (10%) cancer. The other patients (<10% per group) included more than 15 different tumor types, both hematological and solid tumors, at various stages and/or courses of treatment. On average, 1008 mg of IV iron as FCM was administered with approximately 1.0-1.5 g/dL increase in hemoglobin during the observation period (Figure 1). The total dose of iron administered ranged from 100 mg to 4,000 mg. Half of the patients received FCM as single doses of greater than 500 mg iron (with 14 having individual doses of 1,000 mg) with similar effectiveness in correcting anemia as more frequent individual lower doses. Patients that received <500 mg individual doses required approximately 6.5 administrations (average individual dose of 127 mg) whilst those with higher doses had an average of 2 administrations (average individual dose of 606 mg). Adverse events (AEs) were reported in 11 (8%) patients with 1 (0.7%) serious adverse event (SAE). The reported SAE, tachycardia, was considered by the Investigator to be unlikely related to FCM. *Summary/Conclusions.* These are the first prospective observational data of FCM in oncology and suggest a role for IV iron alone in the correction of anemia in ID patients. FCM was administered in many combinations (from multiple low doses to single high doses) with similar Hb responses.

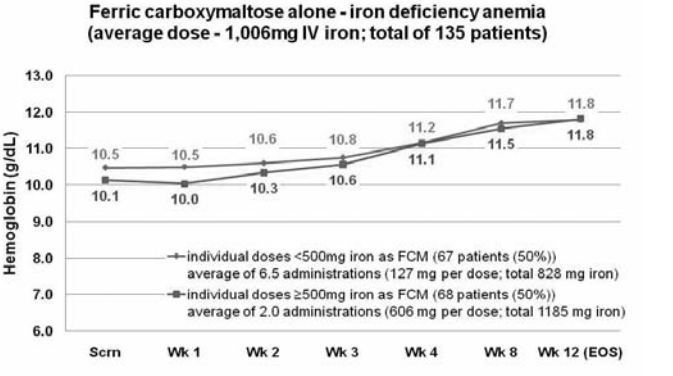

Figure 1. FCM alone in correction of iron deficiency anemia.

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RENAL FUNCTION IN PATIENTS WITH THALASSEMIA INTERMEDIA

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Background. Limited reports on renal function in patients with thalassemia major highlight proximal tubular damage and glomerular hyperfiltration attributed to chronic anemia, iron overload and chelation therapy. Data on renal function in patients with thalassemia intermedia (TI) is lacking. *Aims.* To evaluate levels of serum and urinary creatinine (Cr), and urinary protein in patients with TI and their correlation with several disease-related parameters. *Methods.* This was a cross-sectional study of 50 patients with TI treated at the Chronic Care Center, Beirut, Lebanon. Data on patient demographics, status of the spleen, treatment received, and disease-related complications were collected. Blood samples were obtained for assessment of steady state serum ferritin (SF), non-transferrin-bound iron (NTBI), total (Hb) and fetal (HbF) hemoglobin, nucleated red blood cells (NRBC), platelets, and serum Cr levels. Urinary samples were collected for measurement of spot protein and Cr concentrations. Direct determination of liver iron concentration (LIC) was performed using R^2 magnetic resonance imaging. None of the patients received iron chelation or fetal hemoglobin induction therapy. *Results.* Patient and disease characteristics are summarized in Table 1. None of the patients had systemic arterial hypertension. The mean±SD serum Cr, spot urinary protein, and urinary protein/Cr ratio were 0.48±0.15 mg/dL (range, 0.20-0.90 mg/dL), 269.4±291.7 mg/L (range, 59.0-1588.0 mg/l), and 325.7±282.5 mg/g (range, 93.3-1538.9

mg/g). A total of 24 (48%) patients [mean age 23.5 years, 19 males] had serum Cr levels ≤0.4 mg/dL (i.e., possible glomerular hyperfiltration), and 7 (14%) patients [mean age 27.1 years, 3 males] had a protein/Cr ratio >500 mg/g (macroproteinuria). Among study variables, age was positively and significantly correlated with serum Cr level (rs = 0.487, P<0.001), males had a significantly higher mean serum Cr level than females (0.55 *vs.* 0.43 mg/dL, P=0.002), and splenectomized patients has a significantly higher mean serum Cr level than non-splenectomized patients (0.56 *vs.* 0.46 mg/dL, P=0.031). Urinary protein/Cr ratio had a significantly positive correlation with each of the following: SF (rs = 0.202, P=0.048), NTBI (rs = 0.444, P=0.001), LIC (rs = 0.357, P=0.011), and NRBC (rs = 0.381, P=0.006); yet a significantly negative correlation with Hb level (rs = -0.252, P=0.008). *Summary/Conclusions.* Glomerular hyperfiltration and macroproteinuria are common in patients with TI who have not received iron chelation therapy. These alterations in renal function may portend future risk for progression of chronic kidney disease. Anemia, hemolysis, and iron overload play significant roles towards the development of macroproteinuria in this patient population. Large prospective trials are called for to better understand the natural history of renal function in TI patients and to assign the optimal preventive approaches before overt renal insufficiency develops.

Table 1. Patient and disease characteristics.

Parameter	Value
Mean age ± SD, years (range)	27.3 ± 12.1 (8-63)
Male/Female	22/28
Splenectomized, (%)	39 (78)
Pulmonary hypertension, n (%)	28 (56)
Thromboembolic disease, n (%)	14 (28)
Occasionally transfused, n (%)	15 (30)
Mean Hb[a] ± SD, g/dl (range)	8.3 ± 1.8 (4.9-13.1)
Mean fetal Hb ± SD, % (range)	44.1 ± 29.5 (8.7-100)
Mean platelet count ± SD, x10^9/l (range)	787.5 ± 410.3 (135.0-1733.0)
Mean NRBC count ± SD, x10^6/l (range)	376.73 ± 52.7 (0.0-1629.0)
Mean SF ± SD, ng/mL (range)	985.4 ± 669.1 (18.0-3157.5)
Mean NTBI ± SD, µmol/l (range)	2.9 ± 3.7 (-3.7-10.0)
Mean LIC ± SD, mg Fe/g dw (range)	9.6 ± 7.4 (0.6-32.6)

Hb = hemoglobin; NRBC = nucleated red blood cell; SF = serum ferritin at steady state; NTBI = non-transferrin-bound iron; LIC = liver iron concentration; dw = dry weight.
[a]Pre-transfusion for transfused patients.

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INCREASED LEVELS OF ANGIOGENIC AND INFLAMMATORY CYTOKINES IN PATIENTS WITH THALASSEMIA MAJOR AND DOUBLE HETEROZYGOUS HBS/BETA-THALASSEMIA; REDUCTION OF TNF-α POST-DEFERASIROX THERAPY

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Background. Angiogenesis is a crucial process in the pathogenesis of several disorders. Endothelial damage and inflammation are implicated into the biology of sickle cell disease (SCD) and the beta-thalassemia syndromes. Deferasirox (Exjade®) is a once-daily orally administered iron chelator approved for the treatment of transfusional iron overload. *Aim.* The aim of this prospective study was to evaluate the levels of angiogenic and inflammatory cytokines in patients with thalassemia major (TM) and double heterozygocity of SCD and beta-thalassemia (HbS/beta-thal) with iron overload who received chelation therapy with deferasirox. *Patients/Methods.* Forty-five patients (16M/29F) with TM and 20 patients (7M/13F) with HbS/beta-thal were evaluated. Deferasirox was given for a period of 12 months. Serum levels of angiogenic cytokines, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiogenin (Ang), angiopoietin (Angp)-1 and -2 and of inflammatory cytokines including interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), IL-1α, IL-1β, IL-4, IL-10 and transforming growth factor (TGF)-β1 and β2, were measured at baseline (day 1 of deferasirox administration) and then after 12 months post-deferasirox therapy, using ELISA methodology (R&D Systems, Minneapolis, MN, USA, for angiogenic cytokines and ILs and Diaclone, Bensancon, France for TNF-α, TGF-β1 and TGF-β2). Twenty healthy blood donors of similar age and gender were also evaluated as controls. *Results.* Patients with both TM and HbS/beta-thal had increased levels of all studied angiogenic cytokines compared to controls and reduced levels of Angp-1/Angp-2 ratio (P<0.01 for all comparisons). Patients with HbS/beta-thal had increased levels of Angp-2 (P=0.002) and reduced levels of Angp-1/Angp-2 ratio (P<0.001) compared to TM patients. Both TM and HbS/beta-thal patients had also increased levels of IL-6 (P=0.008), IL-1α (P=0.01), TGF-β2 (P=0.01), IL-10 (P=0.02), IL-4 (P=0.03), and TNF-α (P=0.01) compared to controls. There were no differences between TM and HbS/beta-thal patients regarding inflammatory cytokines. In TM, VEGF strongly correlated with TGF-β1 (r=0.652, P<0.001), platelet counts (r=0.503, P=0.001) and white blood cell (WBC) counts (r=0.484, P=0.002), while Angp-2 strongly correlated with TNF-_ (r=0.536, P=0.001), WBC counts (r=0.430, P=0.008) and platelet counts (r=0.330, P=0.01). In HbS/beta-thal patients Angp-1/Angp-2 ratio strongly correlated with ALT (r=-0.611, P=0.007) and Hb (r=0.498, P=0.036). Twelve months post-deferasirox administration, there was a dramatic reduction of ferritin, SGOT and SGPT compared with baseline values in both patient groups (P<0.0001). TM patients showed a continuous increase of both VEGF (mean±SD: from 680±489 ng/mL prior to deferasirox to 801±547 ng/mL post-deferasirox; P=0.002) and bFGF (from 16.2±17.1 ng/mL prior to deferasirox to 33.1±28.8 ng/mL post-deferasirox; P=0.001) after 12 months of deferasirox therapy. Patients of HbS/beta-thal showed no alterations in terms of angiogenic cytokines that continued to be elevated compared to controls. In terms of inflammatory cytokines deferasirox produced a reduction of TNF-α in both studied groups (P<0.01 for all comparisons). *Summary/Conclusions.* Our study suggests that angiogenic and inflammatory cytokines are increased in patients with TM and HbS/beta-thal and thus they have a role in the pathogenesis of these disorders. Further studies are needed to fully elucidate the effect of deferasirox, if any, on inflammation. Deferasirox seems to have no impact on angiogenic cytokines.

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COMPLICATIONS OF β – THALASSEMIA INTERMEDIA IN PATIENTS REFERRING TO HEMATOLOGY CLINIC OF ALI-ASGHAR CHILDREN HOSPITAL DURING 1996 – 2008

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Background. Beta thalassemia intermedia (TI) is a member of thalassemia family that has a less severe clinical phenotype compared to thalassemia major;TI patients experience many complications, including cardiopathy, pulmonary hypertension, gallstones, splenectomy, endocrinopathies, osteoporosis, etc. *Aim.* Considering the high prevalence of thalassemias in Iran we carried out this study to determine the frequency of TI complications in Iranian patients. *Methods.*Our study was a descriptive cross sectional survey; using the sampling method of "census", we enrolled all the patients with the diagnosis of TI, who had been visited in hematology clinic of Ali-Asghar children's hospital in1996-2008;164 cases were selected and the collected data from their medical records was analyzed by the SPSS software. *Results.* In 164 patients 83 were male and 81 female; mean age of the patients at last visit were 16.5±9.7 years (3-55years),mean age at diagnosis was5.4. Mean hemoglobin was 9.14±1.13,mean Hb F=59, mean serum ferritin was758 ng/mL. 40.2% of the patients were receiving transfusions, either regularly or irregularly; and the mean age at the first transfusion was 7.7±5.4 years; 45.3% and 14.5% had undergone splenectomy and cholecystectomy, respectively;61.9 % had at least one valvular abnormality,pulmonary hypertension was detected in 16%, endocrine complications were detected in 7.6 % ; hypogonadism was the most frequent(4.8),hypothyroidism in 2.1% and no diabetes mellitus; in addition, 50% of the cases were found to have either osteoporosis Z-score ≤-2.5, or osteopenia. *Conclusion.* Because some patients had very few clinical complication and others severe,a careful analysis of the clinical,hematological,genetic and molecular evaluation is necessary specially in young children.

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ERYTHROCYTE PHENOTYPES IN EMIRATI PEOPLE WITH α THALASSEMIA

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Background. Populations with very high frequency of α^+ thalassemia allele have three distinct red cell phenotypes: normal ($\alpha\alpha/\alpha\alpha$), silent

carriers (-α/αα) and α+ thalassemia trait (-α/-α). Although red cells of silent carriers are relatively smaller, they overlap with and are clinically indistinguishable from normal. Thus, two phenotypes are clearly recognized, normal and small red cells. In such populations, the erythroid reference intervals are widely dispersed and skewed to one side. In United Arab Emirates (UAE), one half of the population has α-gene mutations and one third has small red cells mostly due to α+ thalassemia trait. Therefore, separate standards are necessary for the people with normal and small red cells. *Aims.* To determine reference intervals for native UAE citizens with normal and small red cells. *Methods.* In UAE, the population is tribal and endogamous. Hemoglobinopathies are also very common. We studied the erythroid lineage in 1,079 participants in the premarital screening program, which uses complete blood count and hemoglobin electrophoresis to detect hemoglobinopathies. The program is mandatory and fully funded by the government. Subjects with hemoglobinopathies other than α+ thalassemia and those with iron deficiency were excluded from analysis. The frequency distribution of MCV in the remaining 896 subjects was visibly bimodal. Normix program (http://www.alumni.caltech.edu/~wolfe/normix.htm) was used to separate subjects with normal from subjects with small red cells. The small red cell phenotype by diagnostic exclusion was considered α+ thalassemia trait. Hardy-Weinberg equation was used to derive genotype frequencies from the frequency of α+ thalassemia trait, αα/αα genotype. *Results.* The normal phenotype had MCV >78.0 fl (n=715) and the α+ thalassemia trait phenotype had MCV <78.0 fl (n=181). The erythroid indices were significantly different between the two groups (Table). The prevalence of α+ thalassemia trait (0.17), silent carriers (0.48) and normal phenotype (0.35) and the frequency of α+ thalassemia allele (0.4) were all very similar to those found earlier in the same population using genotyping and other phenotyping methods. Among ten largest tribes, the prevalence of α+ thalassemia trait varied between 0 and 0.31. The reference intervals for normal phenotype closely overlapped with those for Caucasians and for normal homozygotes defined by genotyping. The intervals for α+thalassemia trait also closely overlapped with those for α+ thalassemia homozygotes defined by genotyping. *Conclusion.* In populations with very frequent α+ thalassemia mutations, two sets of reference intervals should be determined and used in clinical practice.

Table. Erythrocyte indices in subject with normal (A) and small (B) red cells.*

	N	RBC (x10⁶/mL) Mean ±2SD	Hb (g/dL) Mean ±2SD	Het (%) Mean ±2SD	MCV (fl) Mean ±2SD	MCH (pg) Mean ±2SD	MCHC (%) Mean ±2SD	RDW (%) Mean ±2SD
A	All 715	5.1 4.1-6.2	14.5 11.7-17.4	44.1 35.9-52.3	86.1 77.5-94.6	28.4 24.7-32.1	33 31.1-34.9	11.9 10.5-13.3
	M 398	5.4 4.5-6.3	15.4 13.4-17.5	46.8 40.5-53.0	86.4 78.2-95.6	28.6 25.1-32.1	33.1 31.1-35.0	11.8 10.6-13.0
	F 317	4.8 4.1-5.5	13.4 11.7-15.1	40.8 35.9-45.7	85.6 76.6-94.6	28.1 24.3-31.9	32.9 31.1-34.6	12 10.4-13.7
B	All 181	5.9** 4.6-7.1	13.6** 10.7-16.4	42.6** 33.8-51.4	73** 67.2-78.7	23.2** 20.8-25.7	31.9** 30.2-33.5	12.8** 11.4-14.1
	M 97	6.3 5.4-7.1	14.6 12.8-16.4	45.8 40.1-51.6	73.1 67.2-79.0	23.3 20.9-25.7	31.9 30.2-33.5	12.7 11.4-14.1
	F 84	5.4 4.5-6.2	12.4 10.6-14.2	39 33.7-44.2	72.8 67.1-78.4	23.2 20.6-25.7	31.8 30.2-33.4	12.9 11.6-14.3

* MCV=78.0 fl used as separating value.
** P<0.0001

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AUTOMATED RED CELL EXCHANGE IN PATIENTS WITH SICKLE CELL DISEASE

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The usefulness of red cell exchange (RCE) was tested in diseases such as sickle cell anemia and malaria. In adults with sickle cell disease, it has been used in the treatment of acute chest syndrome, priapism, osteonecrosis, lower limb ulcers, as well as to control painful crises and prevention of strokes. But there is little evidence of its use in patients with sickle cell disease who have more serious comorbidities such as cardiac or renal impairment. We report four cases of patients included in RCE program. *Patient 1.* 17 year-old male from Maurtania homzygous for hemoglobin S, kidney transplant secondary to membranoproliferative glomerulonephritis, with many sickle cell crises that caused acute transplanted kidney failure. For that reason we indicated RCE. He held 19 sessions (every four weeks), with an average hemoglobin S (Hb S) before the procedure of 45% and after the procedure of 23.4%. We used dialysis arteriovenours fistula as access route. *Patient 2.* 37 year-old male diagnosed with sickle cell syndrome (heterozygous Hb S and beta thalassemia minor). Mechanical aortic valve and ascending aortic prosthesis bearer; anticoagulated with acenocoumarol. He had many sickle cell crises that required long periods of admisssion to the hospital for pain management. He underwent 12 sessions of RCE. Mean Hb S before the procedure was 49% and 29% after them. *Patient 3.* 49 year-old woman heterozygous for Hb S with hemochromatosis secondary to transfusion therapy, renal failure, keratoconus and diabetes mellitus secondary to severe iron overload. Ten RCE were held with an average of 34.8% of Hb S before the procedures and 17% after them. *Patient 4.* 51 year-old portuguese female with sickle cell disease, severe pulmonary hypertension and sickle cell hemolytic crises. Six sessions were performed. The average Hb S before the procedure was 38% and 19% after that. Patients entered automated RCE program for the indications above, using the Cobe Spectra system for red cell replacement at intervals of four weeks. ACD-A and heparine were the anticoagulants chosen. The volume to be infused was estimated according to the size and weight of the patient. In the first case we used hemodialysis arteriovenous fistula as access route. In the rest of the patients femoral venous lines were used as they did not have appropiate peripheral vein access. None of them showed any complications during the procedures. *Conclusion.* Automated blood count replacement is a safe and effective procedure in patients with sickle cell disease, even in those with significant comorbidities such as renal, heart or lung disease, where it is important to keep an appropiate body volume management. In all cases low levels of Hb S less than 30% and Hb levels around 9g/dL were hold, which according to the literature prevent the development of stroke in patients with sickle cell disease. A renal graft protection was achieved in the first case. In the same way, a decreased in the quantity and intensity of pain crises was also achieved with less analgesia requirements and a better quality of life for all the patients.

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CARDIAC T2* MRI ASSESSMENT IN TRANSFUSION DEPENDENT ANEMIA OF OUR PATIENTS: PRELIMINARY RESULTS

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Cardiac complications are the major cause of mortality in patients with transfusional iron deposition. Conventional tools such as serum ferritin level, ECG, echocardiography, and even magnetic resonance have been reported as unsatisfactory in defining and evaluating the risk mentioned above. Since the beginning of 2000's, myocardial T2* MRI has been widely used as a critical method for not only demonstration of iron deposition in the myocardium objectively, but also for assessment of prognosis of the patients. In current study, we aimed to evaluate the myocardial iron deposition of our patients with chronic transfusion dependent anemia by their T2* MRI results for the first time. We also overviewed other findings and treatment regimens of the patients, as well as T2* results, which studied between December 2008 and January 2010. Study group included 68 patients: 58 with thalassemia major, 3 with Fanconi aplastic anemia, 2 with thalassemia intermedia, 2 with hereditary sferositosis and 1 with pure red cell aplasia. They were consisted of 30 males (44.1%) and 38 females (55.9%), aged from 8 to 23 years old (mean 14.8±2.1). T2* results were as follow: It was lower than 10 msn in 5 patients (7.4%), between 10 and 20 msn in 33 patients (48.5%), and higher than in 20 msn in 30 patients (44.1%). In patients in whose T2* values were lower than 10 msn, between 10 and 20 msn and higher than 20 msn, mean ferritin levels were 3810±2080, 2125±950, 2451±1024 and mean LVEF were 62.8±4.6, 72.9±6.2, 74.9±4.2, respectively. The patients were treating with combination of Desferrioxamin and Deferipron (47%), Deferrioxamin only (30.9%) and Deferasirox only (17.7%) in terms of treatment options, when the time of T2* images were obtained. Here we discussed effects of T2* results on chelation treatment regimens and prognostic value in patients with iron deposition.

1848

ROLE OF HEPCIDIN HORMONE IN EGYPTIAN PATIENTS WITH B- THALASSEMIA MAJOR

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Bachground. Hepcidin, a hepatic hormone, controls plasma iron concentration and tissue distribution of iron by inhibiting intestinal iron

absorption, iron recycling by macrophages, and iron mobilization from hepatic stores. Thalassemia syndromes represent a clinical setting where hepcidin is regulated by opposing influences of ineffective erythropoiesis and elevated iron load. *Aims.* We aimed to evaluate serum erythropoietin (sEpo) and soluble serum transferrin receptors (sTfr) as indicators of erythropoietic activity and to find their relation with hepcidin concentration in patients with β thalassemia major in a trial to explain its rule in iron metabolism for those patients who have iron overload. *Methods.* This study was carried out in pediatric and clinical pathology departments of Zagazig University Hospitals in the period from March to December 2009. The study included 30 β thalassemia Major (TM) children aged between 4- 10 years with a ratio of 1.5:1(18 male and 12 female) and 10 apparently healthy children, age and sex were matched as a control group. All subjects were studied for pre-transfusion hemoglobin (Hb), reticulocytic index (RI), serum ferritin, sEpo and sTfr, and serum hepcidin level. *Results.* The mean RI, sEpo and sTfr values were significantly higher in the TM group compared to the control group. The results showed that hepcidin concentration was significantly decreased in TM than control group. There was a positive correlation between hepcidin and Hb, while there was a negative correlation between hepcidin and sEpo and sTfr values. On the other hand it did not correlate with indices of iron stores. *Summary and conclusion.* sEpo, sTfr and RI could be used as accurate and reliable indicators of successful erythroid marrow activity and these results underline the role of erythropoietic activity on low hepcidin level in thalassemic patients.

1849

XMN I POLYMORPHISM AND SEVERITY OF BETA THALASSEMIA

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Background. the clinical manifestation of beta-thalassemia syndromes, are extremely diverse and accurate prediction of disease severity based on globins gene mutations alone is complicated by influence on phenotype of genetic modifiers. Among those factors XmnI polymorphism G_ polymorphism is known factor which increases fetal hemoglobin (HbF) production. *Aims.* our aim is to determinate the role of XmnI polymorphism as a factor predicting the severity of the phenotype, and his place in the therapeutic response in a cohort of Algerian patients with homozygote β-thalassemia treated with Hydroxyurea as an inductor of hemoglobin Fetal production. *Methods.* we had studied 65 patients with homozygote β-thalassemia mean age 11±5 years (ranged 3-21). Eleven was intermediate TI and 55 was major TM. we performed gene mutation and determinate Xmn I polymorphism in all patients. Disease severity was assessed based on age of first transfusion, and blood requirement (number of blood units BU per year). We defined good response to Hydroxyurea when a decrease of annual transfusion requirement greater 70% with sustained Hb level above 7g/dL by increasing of HbF level. *Results.* among thirteen mutations identified, three are common IVS1-110 G>A (41%), Codon 6(-A)(23%), Codon 39 C>T (18%), others mutations were occasional such IVS1-1G>A(4,6%) ; IVS1-5G>C ; IVS1-6G>T ; -101 C>T ; IVS2 nt848C>A ; polyA A>G (ATGAA), polyA T>C(ACAA) ; codon30G>C ; ψβ,δ,β et 3'phhf ; D-polya. Homozygosis for a mutation was observed in 33/65 patients, XmnI polymorphism was observed in 31 alleles (24% of patients) with three categories XmnI (+/+), (+/-), (-/-) Codon 6 (-A) was associated with XmnI polymorphism in 80% of cases and just 6% for others mutations. Age of the first transfusion was 31±21 months (4-70) for the category of patients with XmnI (+/+),(+/-), and 20±19 months (4-84) for patients with XmnI (-/-)(p> 0,05). The blood requirement was 10±2 BU/year in the group of XmnI (+/+), (+/-) patients, and was 14±3 BU/y in the group of XmnI (-/-) (p>0, 05). Good response to Hydroxyurea was observed in the group of XmnI (+/+), (+/-) patients (p 0,008), this response was worse in the group of XmnI (-/-) (p0, 0001) (M. Bradai and all. Decreased transfusion needs associated with hydroxyurea therapy in algerian patients with thalassemia major or intermedia, transfusion, 47, 2007). *Conclusion.* the determination of XmnI polymorphism alone seems not a determinant factor of severity of β-thalassemia. However in our experience, it's could bee used as a marker of the response to inductors agents of HbF such hydroxyurea.

1850

EVALUATION OF THE EFFICACY OF INTRAVENOUS IRON SUCROSE FOR TREATING ADULT PATIENTS WITH IRON DEFICIENCY ANEMIA

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Background. Parenteral iron preparations available in the past were associated with a risk of anaphylaxis and death, which made physicians reluctant to use them. The formulation most frequently responsible for these serious adverse events is high-molecular-weight iron dextran. The availability of four other preparations (iron sucrose, ferric gluconate, low-molecular-weight iron dextran, and, more recently, ferric carboxymaltose) with a much better safety profile, is changing the pattern of use of intravenous (IV) iron. *Aims.* The objective of this study was to evaluate the efficacy of IV iron sucrose to treat adult patients with iron deficiency anemia. *Methods.* Between January 2003 and August 2009 we studied 86 adult patients with iron deficiency anemia (IDA). The inclusion criteria were: hemoglobin (Hb) level <12.0 g/dL for women and <13.0 g/dL for men, serum ferritin <12 ng/mL and intolerance or no effect of oral iron therapy (160-200 mg/d over 2 weeks), severe anemia (Hb <7 g/dL) and pregnancy with Hb level <10 g/dL and gestational age over 16 weeks. The main laboratory tests performed were: complete blood cell count, reticulocyte count, serum iron, total iron-binding capacity and serum ferritin. The patients received a weekly dose of 200 mg of iron sucrose until Hb correction or completing the administration of the total dose of parenteral iron recommended for each patient. *Results.* The median age of the patients studied was 42.5 years (age range from 20 to 76). Sixty-nine out of 86 patients (80%) were women. The most common cause of iron deficiency anemia was abnormal uterine bleeding observed in 45.3% of female patients and partial gastrectomy in 53% of male patients. Thirty-five (40.7%) patients were included in this study due to a lack of response to oral iron therapy, 33 (38.4%) showed intolerance to oral iron, 10 (11.6%) presented with a Hb level <7.0 g/dL and 8 (9.3%) were pregnancy. The mean Hb and ferritin values were 8.54 g/dL and 7.63 ng/mL (pre-treatment) and 12.1 g/dL and 99.0 ng/mL (post-treatment) (P<0.0001), respectively. The average increase of hemoglobin were 3.29 g/dL and 4.58 gdL for women and men, respectively. Correction of anemia was obtained in 47 out of 69 female patients (68.1%) and in 12 out of 17 male patients (70.6%). Six patients received blood transfusions before starting intravenous iron treatment. None of the 86 studied patients needed red blood cell transfusions during or after completing the treatment. Only two adverse events [rash (n=1) and palpitation (n=1)] were reported by the investigators among 515 IV iron sucrose infusions. No patient discontinued the treatment. No death was reported by the investigators during the study. *Conclusions.* The use of intravenous iron sucrose is an efficacious and safe option in the treatment of adult patients with IDA who lack satisfactory response to oral iron therapy. This treatment option should be considered mainly in patients with severe anemia in order to obtain rapid increases in the hemoglobin levels and to avoid blood transfusions.

1851

ANEMIA IN ADULT PATIENTS WITH SEVERE FALCIPARUM MALARIA, OUTCOMES AND ASSOCIATED FACTORS

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Background. People in more than 100 countries (subtropical and tropical area) are suffered from falciparum malaria. Anemia is one of the most common complications in severe falciparum malaria patients. The prevalence of anemia in falciparum malaria patients varies from different area. Especially in severe anemia malaria group, the association between anemia and clinical parameters had not been discerned yet. Moreover, there is no consensus on when the most appropriate time to give pack red blood cell (PRC) transfusion is. Since the transfusion reaction may occur, physicians have to weight between the benefit of blood transfusion. Thus, the data on transfusion reaction was not available especially in severe anemia malaria patient. *Aims.* To determine the prevalence of anemia, the proportion of patients with severe anemia (using WHO criteria 2006), outcomes and associated factors of severe anemia, and the prevalence of transfusion reaction among adult patients with severe anemia falciparum malaria at Mae Sot General Hospital, Tak province, Thailand. *Methods.* A retrospective study was conducted in adult patients (age 15 years and older) with severe falciparum malaria according to WHO criteria 2006, admitted to Mae Sot General Hospital between 1st January 2004 and 30th November 2008. Case data were retrieved from hospital records. The ethice committee of Tropical Medicine Institute approved this study. *Results.* 262 patients were enrolled into the study. The majority (72.9%) were male, median age

33 years (range 15-94), and Myanmar (61.8%). The common clinical presentations were fever (99.2%), pallor (50.4%), jaundice (48.9%), Glasgow coma scale (GCS) scores ≤10 (30.7%), and hepatomegaly (20.1%). One hundred seventy six patients (68%) were given the combination of artesunate and mefloquine regimen. The prevalence of anemia and severe anemia (hemoglobin less than 5 g/dL) on admission were 70.2% and 5.3%, respectively. Two-thirds of patients without anemia on admission developed non-severe anemia during hospitalization, making the overall incidence 90.1% (236/262). Logistic regression analysis showed no factor to be independently associated with severe anemia, when compared with cases without anemia. However, the white blood cell and platelet count, and bicarbonate were independently associated with severe anemia, when compared with non-severe anemia on admission (P=0.022, 0.022 and 0.16 respectively). The following factors were independently associated with overall anemia (both severe and non-severe anemia): referred cases (P= 0.01), microcytic red cell (P<0.001), blood urea nitrogen (BUN) (P< 0.001), creatinine (P=0.021), and platelet counts (P=0.02). No patient with severe anemia malaria died. Moreover, patient with non-severe anemia malaria showed high mortality among groups (25.9%). Median number of PRC unit per each patient was 2 units (range 1-18). Patients who received PRC transfusion had longer hospital stays when compared with no PRC transfusion (P<0.001) but none of them developed an acute transfusion reaction during RPC transfusion. *Conclusions.* Overall, anemia was a common complication in severe falciparum malaria, but the rate of severe anemia was low. Factors independently associated with only anemia were BUN, creatinine, and platelet counts, microcytic red cell, and referred cases. No acute transfusion reaction was found among patients receiving PRC transfusions.

1852

CARE OF IMMIGRANT PATIENTS IN A TROPICAL MEDICINE UNIT: USEFULNESS OF SYSTEMATIC SCREENING FOR HEMOGLOBINOPATHIES

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Background. In 2009, more than 142,284 foreign persons live in Almeria, southern Spain, representing a 20.8% of the total population (684,426 inhabitants). The percentage of foreign residents estimated for the entire Spanish territory is around 12%. The Tropical Medicine Unit (TMU) of our hospital was founded In 2004, and it belongs to the Spanish Network of Tropical Medicine Units (REUMT), coordinated by the Carlos III Health Institute. Hemoglobinopathies, both structural and thalassemic, are frequent in black population, due, at least partly, to their protective effect against endemic malaria. *Aims.* To assess the usefulness and effectiveness of initial systematic screening for Hemoglobinopathies in all black patients seen in the TMU. *Materials and methods.* Analytical data from black immigrant patients seen for the first time in the TMU of the Poniente Hospital throughout 2008 were reviewed. Blood samples were analyzed in an ADVIA 2120 (Siemens®) hematological cell counter. As screening for the presence of structural hemoglobin variants, the high-pressure liquid chromatography (HPLC) Hi-AUTO A1c 8160 (Menarini®) system was used. For definitive characterization of abnormal hemoglobins, electrophoresis in agarose gel and acidified medium was ordered. In cases with microcytosis/hypochromia not associated to iron deficiency, HbA2 and HbF were dosified. In case they were normal, genetic analysis for α-thalassemia was carried out when suitable. *Results.* A total 335 immigrant patients coming from sub-saharan Africa were seen in the TMU during 2008; average age 28.3 years (range 15-55); 305 male (91%). The most frequent countries of origin were: Senegal (120 patients, 35,8%), Mali (60, 17,9%), Guinea-Bissau (55, 16,4%), Ghana (23, 6,9%), Mauritania (22, 6,6%) and Gambia (21, 6,3%). The average hemoglobin concentration was 14,9 g/dL (range 8,2-18,7 g/dL). Since the implementation of systematic screening, 46 cases of hemoglobinopathies have been detected. Of them, 26 (56,5%) were Hb AS (sickle cell trait), 10 (21,7%) Hb AC (heterozygous C hemoglobinopathy), 3 (6,5%) homozygous α-thalassemia, 2 heterozygous α-thalassemia, 2 Hb AS+β-talassemia minor, 1 Hb CC (homoozygous C hemoglobinopathy), 1 β-delta thalassemia and 1 Hereditary Persistence of Fetal Hemoglobin (HPFH). Sickle cell trait has been detected in patients coming from all countries, except Burkina-Fasso, Sierra Leone and Ivory Coast. Hemoglobinopathy C was detected in originals from Ghana (4 Hb AC, 1 Hb CC), Mali (4 Hb AC) and Burkina-Fasso (1 Hb AC). The higher percentage of hemoglobinopathy carriers were found in Nigeria (4/7, 57,1%), 2 homozygous alfa-thalassemias, 1 Hb AS and 1 Hb AS+β-thalassemia; Equatorial Guinea (3/7, 42,8%, carriers of Hb AS; and Ghana 7/23 (30,4%) (5 hemoglobinopathies C, 1 Hb AS and 1 HPHF). *Conclusions.* The gradual increase in the proportion of immigrant patients in our area makes it necessary to implement screening programs for hemoglobinopathies, in order to assess the real incidence, define risk groups (pregnant women, newborns) and be able to detect severe forms that would benefit from early treatment. The HPLC system is available in a large number of clinical laboratories for the measurement of HbA1c. It is a fast, simple, affordable and reliable method, and can be of great usefulness in the detection of hemoglobinopathies.

1853

PERIPHERAL QUANTITATIVE COMPUTED TOMOGRAPHY(PQCT) STUDY OF THE FUNCTIONAL MUSCLE-BONE UNIT IN FEMALE PATIENTS WITH β -THALASSEMIA MAJOR

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Background. Osteoporosis, is common in adult patients with β-thalassaemia, relates to expansion of bone marrow and reduction of bone density and constitutes a major cause of bone morbididy. In most studies, osteoporosis is diagnosed on the basis of reduced bone mineral density by means of dual energy x-ray absorptiometry. Peripheral quantitative computer tomography (pQCT) was used recently to assess the changes in volumetric bone mineral density (vBMD) in various bone compartments; however the effect of osteoporosis on bone marrow cavity and muscles has yet to be explored. *Aims.* To evaluate the muscle and bone cross-sectional area, along with the cortical bone density, thickness and strength by means of pQCT in a group of female patients with β-thalassemia major and compare with an age matched control group in order to assess possible effects of muscle over bone. *Methods.* Twenty-six female patients with β -thalassemia major (age 22-41, body-mass index/BMI: 21-23) and history of fragility fracture over a 1-year period were enrolled in an open perspective study, performed in a two-year period. The control group was composed of 25 females healthy volunteers, matched for age and BMI with the patient group; all the control subjects were free from bone diseases. The study was conducted with the approval of the hospital ethics committee and informed consent was obtained from all of the human subjects. The pQCT measurements were performed at the 14%, 38% and 66% of the non-dominant tibia length. Cortical bone density (CBD), cortical thickness (CTH),marrow cavity area(MC) strain-stress index (SSI), bone cross-sectional area (BCA) and muscle cross-sectional area (MCA) were calculated. *Results.* CBD, CTH, BCA ,MCA and SSI were reduced in the β -thalassemia major females in comparison with the control subjects (P<0,001). MC of female patients were significantly higher compared with healthy female controls (P<0.01). *Conclusions.* Women with β-thalassemia major showed poor quality (low CBD, CTH) fragile bones (low CTH,BCA,SSI) with expanded marrow cavity and low adaptive response of bone to muscle force.

1854

X-LINKED SIDEROBLASTIC ANEMIA (XLSA): TWO NEW MUTATIONS IDENTIFIED IN MALES

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Sideroblastic anemias (SA) are a heterogeneous group of disorders, congenital or acquired, associated with abnormal heme biosynthesis, characterized by: 1) microcytic hypochromic anemia, with dimorphism, 2) presence of ringed sideroblasts in the bone marrow, 3) important secondary hemosiderosis. Phenotypic expression of XLSA varies considerable in males. Most frequently, it is diagnosed during the first years of life and is associated with mutations (primarily missense) in the erythroid-specific 5-aminolevulinic acid synthase gene, ALAS2 (Xp11.21). In the less severe forms, the diagnosis may be delayed to the first two decades of life or to the middle age, when the secondary iron overload is remarkable. Females can also have severe anemia, particularly after the fourth or fifth decade of life, due to unbalanced X chromosome lionization. Depending on the ALAS2 mutation location, some patients may

respond to oral pyridoxine treatment. Severe cases are transfusion dependent. *Case 1.* A 42-year-old male was referred to elucidate a chronic microcytic hypochromic anemia: Hb 8,7 g/dL, VGM 55 fL, HGM 17 pg, RDW 30% and peripheral blood smear with dimorphism. Serum ferritin 2932 ng/mL (normal 18-370), transferrin saturation 89% and 20% of ring sideroblasts in the bone marrow. A diagnosis of XLSA was made and treatment with 150 mg/d pyridoxine raised the hemoglobin level 1,5 g/dL in 3 weeks. Molecular analysis of ALAS2 gene showed a not previously described missense mutation at codon 147 (CGC-CAC) (Glu147His) in hemizygous state. *Case 2.* An 8-year-old boy presented with microcytic hypochromic anemia: Hb 10.9 g/dL, VGM 67 fL, HGM 20 pg, RDW 17% and peripheral blood smear with anisocytosis and anysochromia but without observable dimorphism. Serum ferritin 70 ng/mL (normal 18-370), transferrin saturation 19%. His mother presented a mild normochromic and normocytic anemia (Hb 11.1 g/dL, VGM 84.8 fL, HGM 28 pg) contrasting with a high RDW (26%). Two erythrocyte populations were observable in peripheral blood smear. Histogram confirmed a marked dimorphism where 17,6% of the cells were microcytic and 22,6% hypochromic. Each one of the two populations had his own reticulocyte counterpart. Serum ferritin (451 ng/mL) was indicative of iron overload. A diagnosis of XLSA was made and after twelve months of pyridoxine treatment no increment on haemoglobin level was observed. Molecular analysis of ALAS2 gene showed a not previously described missense mutation at codon 503 (GCC-GTC), resulting in the Ala503Val amino acid change, in hemizygous state. The mother was heterozygous for the mutation. To demonstrate that these new mutations were not polymorphisms, we screened 100 DNA samples from unrelated females and not find any allele with these nucleotide substitutions. *Conclusion.* Considering that XLSA phenotype severity varies considerable in males, and can also be present in females, all patients who are suspected as having XLSA or at-risk individuals in families with XLSA should have their DNA examined for ALAS2 mutations, irrespective of their haematological findings, sex and age. Identification of the ALAS2 mutation allows an early treatment with pyridoxine and prevention of iron overload and the identification of heterozygous women in family who will benefit from genetic counselling.

1855

EVALUATION OF GENERAL EXAMINATION OF BLOOD AS PROGNOSTIC INDICATOR IN THE GENERAL POPULATION OF DEPARTMENT OF URGENT INCIDENTS

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Background-Aim. The hematological parameters of patients that arrive in the surgeries of Department of Urgent Incidents (DUI) the Hospitals - Centres of Health were correlated with the mortality inside the week. Even if there are various systems of grading the prognostic value of various laboratorial examinations, the hematological parameters do not correlated yet with particular prognostic role. *Material and Method.* 514 possessed patients (286 men and 228 women) that arrived in the DUI of our hospital and then hospitalized more than 7 days, were retrospectively studied - independent the cause of entry. The rate of mortality was 6,42% (33 individuals), while the means age of subsistent and decedent were 59,7 and 78,2 years, respectively. The values of haematological parameters were recorded and were statistically analyzed in order to find possible correlation between those values and the final result of their situation inside the week. *Results.* As you can see at the tables 1 and 2.

Tables 1 and 2.

	SUBSISTENT		DECEDENT		P
	Men	Women	Men	Women	
WHITE (109/L)	8,7	8,6	10,2	12,9	<0.0001
Hb (g/dl)	13,8	12,6	11,8	11,5	Men<0.0001 Women=0.218
PLT (x109/L)	267,1	291,7	291,4	252,6	0,679

	WBC			PLT			Hb Men			Hb Women		
	N.R.	A	M	N.R.	A	M	N. R	A	M	N. R	A	M
SURVIVAL: N	212	228	31	441	13	27	163	2	121	173	7	48
DEATH: N	9	19	5	24	4	5	6	0	13	9	1	4

[where: N.R.=normal range (White cells=4000-10000, Plt=150000-450000, Hb men=14-17,5 and Hb women=12,1-15,7 A=Increased value (White cell>10000, PLT>450000, Hb men>17,5 and Hb women>15,7 and M=Reduced value (White cells<4000, PLT<150000, Hb men<14 and Hb women<12,1)]

1856

COMPOUND HETEROZYGOSITY FOR HB C/ + β-THALASSAEMIA.A CASE PRESENTATION

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The Hb C is due to a replacement of the glutamic acid by lysine at point 6 of the β-chain (β,6 Glu-Lys). It is encountered mainly in the Western and South Africa, in Canada and also Italy, Sicily, Turkey and Greece. It exists at the heterozygous form, the homozygous form and the compound heterozygous form in combination with thalassaemia and other haemoglobinopathies. Combined with β-thalassaemia although rare, presents great clinical moderation. We report the case of a 22-years-old soldier of Muslim origin from Thrace, who was referred for investigation due to hypochromic, microcytic anaemia and splenomegaly. Clinical examination showed a slight increase in splenic size. The haematological data of the propositus was: Hb: 11.7 gr/dl, Hct: 38.4%, MCV: 65.4 fl, MCH: 19.9 g/dL, RBC: $5.880\times10^3/\mu L$. Ferritin levels were 67 ng/mL. Microscopic examination of a stained peripheral slide revealed intense hypochromia, severe anisocytosis, microcytosis, baseophilic stippling and target cells. No erythroblasts were found. Osmotic resistance of the red cells was elevated and sick ling test was negative. HPLC hemoglobin variants analysis showed Hb A2: 5.3%, Hb F: 1.8% and an abnormal haemoglobin 83.9% at time 4.88 minute. Electrophoresis of the haemoglobin at alkaline and acid pH showed properties of a compound heterozygote for Hb C and β-thalassaemia. Further molecular screening revealed an IVS II- 745 (β+) thalassaemic mutation. Compound heterozygosity for Hb C and β-thalassaemia is rare and was first described in an Afro-American patient. It has also been described in Italians, North Africans, Turks, Sicilians and Tunisians. The interesting feature of the haemoglobin C β-thalassaemia is the remarkable clinical heterogeneity and differences in haematological manifestations. Although reports on the molecular level are limited there are sufficient data that the variability of the clinical and haematological manifestations reflects the variability of the β-thalassaemia gene which is interacting with Hb C. In our Haemoglobinopathy Prevention Unit whish covers all over Northern Greece during the last 22 years (1986-2007) 80.401 subjects have been examined for haemoglobinopathy and thalassaemia We found 18 subjects heterozygotes for Hb C, one homozygote for Hb C and this one case presented with compound heterozygosity of β-Thalassaemia and Hb C. According to the literature we have not found such a case of a compound Hb C and IVS II- 745 (β+) thalassaemic mutation reported. Our patient seems to have mild anaemia that did not cause any symptoms and was diagnosed during the military service. Detection of thalassaemia and structural hemoglobin variants is important in clinical laboratories in countries with a high percentage of carriers, as Greece. Knowledge of the epidemiology of haemoglobinopathies provides important information for public health planning and appropriate counselling of couples.

1857

SEVERE ANEMIA IN CHILDHOOD: WHAT ABOUT THE REALITY?

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Background. According to the WHO, severe anemia is a public health problem and it is not often studied in the industrialized countries. The main causes affecting about 598 millions of children around the world are iron deficiency, parasitic infections, chronic diseases, nutritional deficiencies and haemoglobinopathies. The aim of our work is to review children who have a hemoglobin level less than 8 g/dL, diagnosed at the outpatient clinic or at the emergency department and to analyze their characteristics. *Material and methods.* This is a retrospective monocenter study that reviews 94 medical records of children aged 0 to 18 who were seen at the Brussels University of Children Reine Fabiola with a hemoglobin level less than 8 g/dL between January 2006 and June 2008. The data were divided into two groups: sickle cell disease (group I) *vs.* other causes of anemia (group II). They are also separated into 2 classes of age, more or less than 6 years old. *Results.* 94 children were included. Among them, 43.6 % suffer from sickle cell disease and 27.7% from e.g. iron deficiency. Furthermore, no diagnosis is observed for 5 % of the cohort and 16% of the children are lost from follow up. 35.8% of group II had a consultation with an hematologist and 64.2% were hospitalized. 42% were coming from Central Africa, mostly Sickle Cell disease patients. The average level of hemoglobin

among the patients is 6.85 g/dL (±1.1) with an average MCV of 77, 2 fL (± 16.4) and an average MCHC of 32.1 g/dL (± 3.8). *Discussion.* Every nine or ten days, a new patient with severe anemia was diagnosed. However there is a recruitment bias because the HUDERF is a pediatric reference hospital. Globally, the pediatric hematologists are taking in charge of chronic children pretty well. Unfortunately, there are failures in the practical guidance of the others when there are some elements in their medical story like prematurity. It could take time before these children achieve to the good place. Furthermore, iron deficiency is the most important cause of anemia, easily identifiable. An interesting point to underline: children older than 6 years and without sickle cell disease, were significantly smaller (≥ 2 standard deviation) than control children of their same age, suggesting an additional underlying cause. *Conclusion.* In our study, near the half is suffering from Sickle Cell disease and a third from iron deficiency. The care for anemic patients with defined underlying cause is pretty good because they are rapidly referred to hematology-oncology department. Nevertheless, for the others, the problem is obviously under recognized and under treated. That is why we recommend redefining the medical approach of children suffering from severe anemia without a clinically known etiology.

1858

HAEMOGLOBINOPATHIES SCREENING IN INMIGRANT AFRICAN POPULATION AT THEIR ARRIVAL TO CANARY ISLANDS

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Structural haemoglobinopathies are one of the most important health problems all over the world. Its incidence has arised in our country in the last years due to inmigration. Canary Islands are one of the main ways of entrance to Subsaharian African and Magreb immigrants. An ordinary study of haemoglobinopathies and thalassemias is not made at their arrival. The aim of this study is to analyze the prevalence of haemoglobinopathies and talasemia in inmigrant population who go to a foster home at their arrival to Gran Canaria, with the aim to detect people with these illnesses or asymptomatic carriers. *Patients and method.* We analyze 155 samples of African individuals who have recently arrived to the island by High performance liquid chromatography (HPLC) methods using an automated D-10 HPLC system (Bio-Rad®). The obtained results were analyzed together with demographic, hematologic and biochemical data. Also molecular studies of the samples were performed. Informed consent was obtained. *Results.* 20 structural haemoglobinopathies were detected (12,9%). 19 of them were heterozygotous (12 HbS and 7 HbC) and one homozygotous (HbSS). 19 samples belonged to subsaharian people and one to a person coming from the Magreb. Most of haemoglobinopathies carriers did not show anemia (84%), nor microcytosis (58%). Genetic advice was given to these haemoglobinopathies carriers in order to minimize the occurrence of homozygotous individuals. *Conclusion.* Structural haemoglobinopathies prevalence observed in our area is ten-fold higher than the one observed in the rest of Spain and in other studies carried out among inmigrant population in our country. According to this data, it seems that screening health programs for haemoglobinopathies and thalassemias in this population should be performed at their arrival to Canary Islands.

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1859

A COMPARISON OF NOVEL SERUM MARKERS FOR THE ASSESSMENT OF LIVER FIBROSIS IN PATIENTS WITH BETA THALASSEMIA MAJOR TREATED WITH DEFERASIROX

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Background. Iron overload enhances oxidative stress within the liver, which is associated with the development of liver fibrosis. Without effective treatment at an early stage, reversible hepatic fibrosis progresses to irreversible cirrhosis. Eighty seven beta-thalassaemia major patients were enrolled in Turkey in the ICL670A0107 study, an international one-year, phase III trial, and were randomized to deferasirox or desferrioxamine. At the completion of the core phase, those who gave consent continued into the extension protocol (ICL670A0107E) that lasted for up to 4 years. *Aim.* To evaluate the effects of up to 5 years deferasirox treatment on liver iron content and fibrosis scores, and the change in liver histology in relation with serum fibrosis markers in beta-thalassaemia patients. *Methods.* This study was run in Turkey following the completion of the ICL670A0107E extension. beta-thalassaemia major patients who completed core phase and continued with deferasirox or switched to deferasirox during 4 year extension were included after consenting for participation in this locally run study. Deferasirox dose was initially based on liver iron concentration at start of treatment; dose adjustments based on monthly serum ferritin and safety trends. Frozen serum samples and liver biopsy specimens which were collected at baseline, 1st year and end of study and stored during the ICL670A0107 core and extension protocols of those patients was used. Liver iron concentrations (LIC) and liver fibrosis scores according to Ishak system were obtained. Serum tenascin, tissue inhibitors of metalloproteinase (TIMP-1), and matrix metalloproteinase (MMP) levels were measured with commercial enzyme-linked immunoassay (ELISA) kits. The same cohort was also stratified according to liver fibrosis (F≤4 *vs.* F>4). *Results.* 66 patients who received deferasirox (n=41) since the core study and switched to deferasirox (n=25) after 1 year and completed 4 year extension study were included. LIC significantly decreased from baseline (21.2±1.6) at 1sty (14.6±1.2) and the EOS (9.4 ±1.0) (P<0.001) while fibrosis score did not differ significantly. In respect of serum fibrosis markers, there were no significant differences in mean serum levels of tenascin, collagen IV and MMP-1 between patients with mild to moderate (F≤4) and advanced liver fibrosis (F5-F6), while levels of TIMP-1 were higher in the latter group both at baseline and end of the 5th year (160.44±9.89 *vs.* 214.87±24.2 μg/L; P=0.047, 71.61±5.89 *vs.* 94.9±4.61 _g/L P=0.012). Only TIMP-1 was also significantly correlated with histological fibrosis at baseline, 1 year, and end of the 5th year (r = 0.31, P=0.048; r=0.32, P=0.02 and r=0.38, P=0.02, respectively). Further, during treatment all serum fibrosis markers decreased significantly compared to baseline irrespective of the fibrosis score. *Conclusions.* This is the first large retrospective cohort design study, to report up to 5 year data of any chelator with respect to effect on LIC, the progression of fibrosis and regulation of hepatocyte regeneration. We show here that matrix derived fibrosis marker TIMP-1 correlates with histological fibrosis and may play an important role in the regulation of hepatocyte regeneration. Serum fibrosis markers significantly decreased from baseline after long-term DFX therapy.

1860

EFFICACY OF DEFERASIROX IN PATIENTS WITH THALASSAEMIA MAJOR IS NOT AFFECTED BY A HISTORY OF HEPATITIS B OR C

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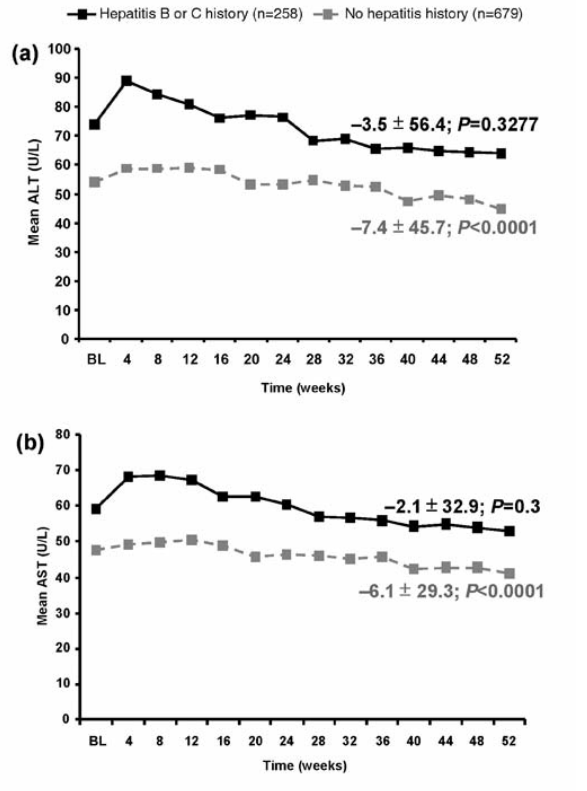
Background. Data suggest that iron overload in thalassaemia major (TM) patients with hepatitis B or C infection increases the risk of liver fibrosis progression. As deferasirox treatment is uncommonly associated with transaminase elevations suggestive of hepatitis (0.3%), it is important to assess whether viral hepatitis status affects the safety profile of deferasirox. Data are reported from patients with a history of hepatitis B or C enrolled in the prospective, multicenter EPIC study. *Aims.* To assess the overall and liver safety of deferasirox in TM patients with/without a history of hepatitis B or C (as recorded at baseline) over 1 year. *Methods.* Transfusion-dependent TM patients aged ≥2 years with

serum ferritin (SF) levels ≥1000, or <1000 ng/mL with history of multiple transfusions (>20 transfusions or >100 mL/kg red blood cells), and MRI-confirmed LIC >2 mg Fe/g dw, were enrolled. Deferasirox starting dose was 10-30 mg/kg/day depending on blood transfusion frequency. Dose adjustments (5-10 mg/kg/day [range 0-40]) were performed every 3 months based on SF trends and safety markers. Safety was evaluated through recording adverse events (AEs) and monthly blood chemistry. *Results.* Of 937 patients with TM, 258 (27.5%) had a history of hepatitis B or C. Patients with hepatitis history were older (mean 26.5±9.9 *vs.* 15.3±9.5 years), more commonly splenectomized (50.4% *vs.* 29.9%), received more transfusions (mean 195 *vs.* 188 mL/kg) and longer duration of chelation at baseline (mean 15.9±10.5 *vs.* 8.7±7.4 years) than those without hepatitis history. Among patients with hepatitis history, 212 (82.2%) completed 1 year of treatment *vs.* 636 (93.7%) without. Most common drug-related AEs in patients with *vs.* without hepatitis history, respectively, included diarrhoea (14.7% *vs.* 6.2%; P<0.0001), abdominal pain (7.4% *vs.* 4.7%; P=0.110), nausea (5.0% *vs.* 3.5%; P=0.291) and skin rash (10.5% *vs.* 13.5%; P=0.205). Three patients (1.2%) with hepatitis history had two consecutive alanine aminotransferase (ALT) increases 10 x upper limit of normal (ULN); all had elevated baseline levels (mean 95.6 U/L). Two patients (0.3%) without hepatitis history had similar increases; one had an elevated baseline level (225 U/L). Mean ALT and aspartate aminotransferase (AST) levels decreased in both groups, significantly in patients without hepatitis history (Figure).

Figure. Mean (a) ALT and (b) AST levels in thalassemia major patients with or without a history of hepatitis B or C.



Serum creatinine was >33% above baseline and >ULN on two consecutive visits in 12 (4.7%) patients with and 25 (3.7%) without hepatitis history. Overall median SF decreased from baseline (3157 ng/mL) by 129 ng/mL after 1 year (P=0.0007) at a mean actual dose of 24.2±5.6 mg/kg/day and mean transfusional iron intake of 0.43±0.2 mg/kg/day. SF reductions were similar in patients with/without hepatitis history (-113 and -148 ng/mL, respectively). *Conclusions.* Deferasirox was well tolerated in TM patients, irrespective of hepatitis history, with no significant differences in incidence of AEs and ALT increases. Baseline liver transaminases were higher in patients with hepatitis history compared with those without, but decreased in both groups suggesting deferasirox improved liver function through iron removal. Reductions in SF were similar in both groups. Further confirmatory and long-term data are required to characterize the effects of deferasirox in patients with viral hepatitis.

1861

N-ACETYLCYSTEINE REDUCES OXIDATIVE STRESS IN SICKLE CELL PATIENTS

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Background. Oxidative stress is of importance in the pathophysiology of sickle cell disease (SCD). Plasma and erythrocyte levels of the major anti-oxidant glutathione are decreased in SCD and treatment with N-acetylcysteine (NAC), the rate limiting precursor for glutathione formation, has been demonstrated to increase glutathione levels and decreased dense cell formation in sickle cell patients.

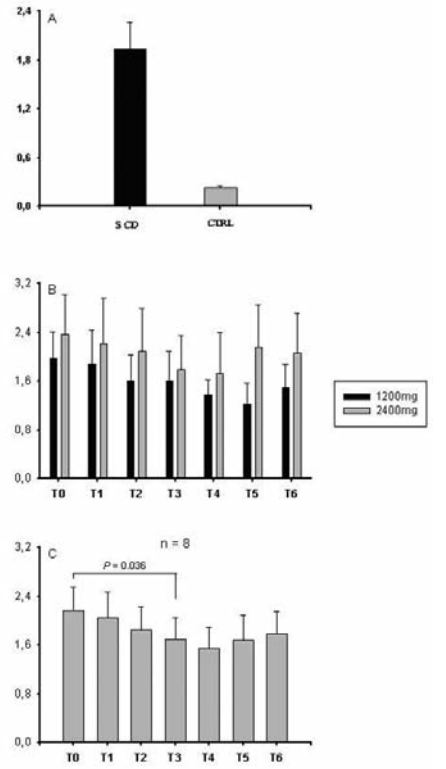

Figure. Erythrocyte phosphatidylserine (PS) expression in sickle cell patients (SCD) compared to healthy controls (CTRL) and during the study period. (A) Sickle erythrocytes (SCD) have a significantly higher outer membrane PS expression than erythrocytes of race matched healthy controls (CTRL) ($P < 0.0001$). (B) PS expression decreases in both 1200 mg (black bars, n = 4)) and 2400 mg (gray bars, n = 4) groups during NAC treatment, returning towards baseline values after cessation of NAC intake. The differences were not statistically significant. (C) Differences in PS expression shown for both NAC dose groups together, where the difference between baseline and 6 weeks (last day of NAC treatment) was statistically significant (Wilcoxon signed ranks test). Sampling performed at 2 weeks interval starting at baseline (T0). Bars indicate means ± SEM.

Figure. Erythrocyte phosphatidylserine expression.

Aims. In this open label randomized trial the effect of oral N-acetylcysteine (NAC) on oxidative stress, coagulation and endothelial activation was studied. *Methods.* Eleven consecutive patients (10 HbSS, 1 HbSβ0-thalassemia) were randomly assigned to treatment with either 1200 or 2400 mg NAC daily during 6 weeks. *Results.* Three patients were excluded due to incompliance. Whole blood glutathione levels increased and erythrocyte outer membrane phosphatidylserine exposure and plasma levels of advanced glycation end-products (AGEs) and cell-free heme decreased after 6 weeks of NAC treatment in both 1200 mg (n=4) and 2400 mg (n=4) groups. NAC treatment did not cause changes in markers of coagulation activation and endothelial activation. During the study period none of the patients experienced painful crises or other SCD or NAC related complications. *Conclusions.* Short term N-acetylcysteine treatment of sickle cell patients seems to reduce SCD related oxidative stress and hemolysis.

1862

IRON OVERLOAD IN PATIENTS WITH CHRONIC HEPATITIS C: A RANDOMIZED CONTROLLED STUDY ON EFFICACY AND TOLERABILITY OF IRON DEPLETION BEFORE ANTIVIRAL THERAPY BY PEG-IFN _2B + RIBAVIRIN

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Background. About 30-39% of patients with chronic hepatitis C (CHC) is affected by iron overload. These patients have a low SVR rate to IFN or IFN + ribavirin (RBV) therapy. No study has investigated whether iron depletion before pegylated IFN or combination treatment improve SVR rate. *Aims.* We have evaluated whether blood letting affect efficacy and tolerability of PEG-IFNalpha2b + RBV in CHC patients. *Methods.* Patients with CHC and ferritin >100 ng/mL were enrolled in the study, excluding homozygotes for HFE mutations and alcohol abusers, and randomized to: 1) repeated phlebotomies up to obtaining a ferritin level <50 ng/mL followed by PEG-IFNα2b + RBV at standard dosage (active arm, AA); or 2) PEG-IFNα2b + RBV at standard dosage (control arm, CA). Primary endpoint was SVR rate; secondary endpoint was frequency of clinical and laboratoristic grade 3-4 adverse events. *Results.* 33 patients (18 naive, 29 genotype 1-4) were enrolled in the study (19 AA, 14 CA). Patients in the two arms did not differ at baseline for any parameter. AA patients underwent a median of 5 phlebotomies, which significantly reduced ferritin, transferrin saturation, ALT and hemoglobin levels, increased platelet count, and had no influence on HCV-RNA level. After a median of 31 days, patients started combination treatment. Ferritin levels increased in both arms during antiviral treatment but remained significantly lower in AA *vs.* CA at each time point. SVR was 31.6% in AA and 21.4% in CA (P=0.698) (difference of efficacy: +10.2% (95%CI -20.59 +40.89%)). Considering only the 18 naive patients, SVR was 60.0% in AA *vs.* 25% in CA (P=0.188) (difference of efficacy: +35% (95% CI: -11.19 +81.19%)). Tolerability, drug dosing reduction or withdrawal were similar in the 2 arms. *Conclusions.* Our results do not indicate that phlebotomies increase efficacy of antiviral therapy in patients with iron overload. However, the strong trend of higher SVR in naive patients undergoing phlebotomies (this subgroup analysis was defined a priori) warrants investigation in large randomized trials focused on them.

1863

FIRST CASE OF HB J-CAMAGUEY ASSOCIATED WITH ALPHA 3.7 IN SPANISH POPULATION

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Background. Structural hemoglobinopathies are the most common monogenic disorders in the world. It have been described about 800 variants of hemoglobin (Hb). However, most of them are clinically silent because they do not produce an alteration in stability, solubility or function of Hb. Nevertheless they are easily identifiable by electrophoretic and chromatographic methods to determine a change of electric charge on the surface of the molecule. *Aims.* The purpose of this communication is the molecular characterization of Hb J-Camaguey [_141(HC3)Arg->Gly]. *Methods.* The propositus is a 70-years-old Spanish woman from Navarra which was referred to us because in a routine study an abnormal band was detected by HPLC Variant®. Hematologic data obtained by an automatic counter of red cells (Coulter GEN-S, Coulter, Hialeah, FI, USA) were in the normal range, with a slight microcytosis (MCV 75.3 fL). The Hb variant was separated by high performance liquid chromatography (HPLC-VARIANT™ BioRad Laboratories, Hercules, CA, USA) and the globin chains by reversed phase HPLC. For the molecular study was necessary a genomic DNA extraction from peripheral blood leukocytes, employing a Bio-Robot EZ1. Alpha gene deletions were studied by α-thalassemia StripAssay and point mutations were studied by automated DNA sequencing with BigDye v1.1, specific for α2 and α1 genes. *Results.* It was observed a 3.7 kb deletion in one allele; and, in the other allele, a point mutation CGT-> GGT in codon 141 of the 3rd exon of α2 gene. The amplification was performed with primers P1A (5'-162810-162829-3') and C3 (5'-163738-163757-3'), this one, specific to the 3' region of the α2 globin gene. *Conclusions.* Hb J-Camaguey [α141(HC3)Arg->Gly] was described the first time in a Cuban family with spanish ascendants and later identified in several families of Chinese origin. All of them in heterozygous state and in one case associated with a α thalassemia deletion (SEA). In these cases had not been determined which of the two α genes was located the mutations. But in our case, this mutation was localized in the α2 gene using a primer specific of the 3' UTR region of this gene. In this case the association with a 3.7 kb deletion which determines the formation of a α2/α1 fusion gene in the other allele, this makes the mutation is observed as an homozygous although it was an heterozygous, cause the other allele not amplified.

1864

HEMATOGICAL PROFILE COMPARATIVE STUDY OF PATIENTS WITH HEART FAILURE

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Background - Aim. To study the variance of hematocrit (Ht), white blood cells (WBC), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), fibrinogen (Fib) and blood immunoglobulins between patients suffering from ischemic cardiomyopathy (IC) and those suffering from dilated cardiomyopathy (DC). Also to study their potential prognostic value in any case, given the fact that in the relative literature it is mentioned that anemia affects the course and evolution of the disease, in patients with congestive heart failure, while in these cases ESR is usually low. *Material - Method.* A total of 86 patients were studied (71 male and 15 female), suffering from heart failure, who presented dilation and left ventricle malfunction and ejection fraction <35%. 49 patients (39 male and 10 female) suffered from DC and 37 (32 male and 5 female) from IC. In these patients, who were under frequent observation for more than one year, the values of Ht, WBC, ESR, CRP, Fib and immunoglobulins were determined. Before this measurements took place, other possible coexisting conditions who could affect the results, such as malignancies, hematological diseases, injuries, gynecological diseases, were excluded. *Results.* Hematocrit, white blood cells and immunoglobulins presented higher values in DC, compared to IC, though there was no statistically important difference between them. CRP and Fibrinogen values were higher in IC compared to DC, though there was no statistically important difference between them. On the contrary, ESR was fairly increased (P<0.001) in the cases of IC (25.3+/-14.1), compared to DC (10.4+/-9.8). *Conclusions.* It is proven, therefore, that there is no statistically important difference between IC and DC, concerning the values of the mentioned lab parameters (who are within the normal range). ESR was an exception, fairly low values (<10) were documented in cases of DC (without a poor prognosis). The other exception was Ht, low values were documented in IC cases. In these cases the low Ht represents an unfavorable prognostic factor, which should not elude our attention.

1865

QUALITY OF LIFE OF SCHOOL AGE THALASSEMIC CHILDREN AT ZAGAZIG CITY

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Background. The assessment of quality of life (QOL) in children especially in children with chronic illness such as thalassaemia is particularly important. An assessment of QOL differs from other forms of medical assessment in that it focuses on the individuals' own views of their well-being and assesses other aspects of life, giving a more holistic view of well-being. *Aims.* The present study was aimed to assess the quality of life of school-age children with thalassemia at Zagazig City. *Methods.* A descriptive study was conducted on a sample of 100 school-age thalassemic children at out-patient Hematology clinic at Zagazig University Hospitals in Sharkia Governorate, Egypt from July 2008 to April 2009. Two tools were used to collect the necessary data. The first was a structured interview questionnaire sheet about socio demographic data of children and their parents as well as medical history. The second tool was a standardized tool (the Pediatric Quality of Life Inventory TM Version 4.0). *Results.* Regarding the total QOL, results of the current study showed that less than half of the studied children (37%) had good score and 58% had fair score according to child report while, in parent report 21% of children had good score and 64% had fair score. The emotional function-

ing score was the lowest (60.2±20.1) followed by physical (63.13±17.8), school (74.95±16.5), and the highest was social functioning (84.15±12.5). There was a significant association between the total QOL of the studied thalassemic children and compliance with blood transfusion and regular iron chelation therapy in both child and parent report. We found that there was no significant association between the total QOL and gender, residence, family income and the educational level of parents while there was a negative statistically significant correlation between the total Qol and birth order. Statistics showed an agreement between both child and parent report. *Summary/Conclusions.* Thalassaemia has a negative impact on perceived physical, emotional, social and school functioning in thalassaemic patients.To improve QOL of thalassemic patients, suitable programs aimed at increasing children's adherence to the treatment regimen and providing psychosocial support are important.

1866

THE INCIDENCE OF THE SENSORINEURAL HEARING LOSS AND OCULAR TOXICITY IN β-THALASSEMIA PATIENTS RECEIVING DESFERRIOXAMINE CHELATION THERAPY

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β-thalassemia is an inherited disorder frequently seen in our country. Regular blood transfusion used for the treatment causes iron overload and tissue damage. To remove excess iron in patients requiring long-term transfussions, chelation therapy is used since 1960's.The first step in chelation therapy is desferrioxamine. The effectiveness of desferrioxamine to control the body iron load is proven in many of the clinical trials. The commonly seen dose limiting side effects of desferrioxamine therapy is sensorineural hearing loss and ocular toxicity. However it is shown in many clinical trials that this side effect is usually reversible, it can lead to tapering the dose and even to stop the therapy. To determine the frequency of eye and auditory complications and their relationship to drug dosage and iron stores in patients receiving desferrioxamine, we studied 52 regularly transfused patients who received desferrioxamine by subcutaneous or intravenous infusion. All enrolled patients underwent otologic and visual assessments. Although the reported incidence of sensorineural hearing loss and ocular toxicity in transfusion-dependent patients receiving desferrioxamine therapy varies from 3.8-40% we have observed no toxic effects in any of the patients. We conclude that the low doses of desferrioxamine such as mean avarage of 40 mg/kg/day given in our patients and also nightly subcutaneous infusion instead of intravenous infusion is relatively safe and limites the side effects like ocular toxicity and hearing impairment.

1867

TISSUE IRON OVERLOAD IN MULTIPLE TRANSFUSED PATIENTS WITH RARE ANEMIAS: AN MR IMAGING STUDY

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Background. MRI has been employed for the non invasive quantification of iron overload and, most recently, for the assessment of iron distribution, in patients with beta thalassemia. However, assessment of transfusional iron overload in rare congenital anemias, other than hemoglobinopathies by means of MR imaging has yet to be described. *Aims.* We aim at a) correlating MRI measurements expressing tissue iron overload, obtained by different MR techniques, with serum ferritin (b) investigating the correlation between the degree of hepatic, splenic, pancreatic, vertebral bone marrow (VBM) siderosis, in multiple transfused patients with rare anemias. *Methods.* Signal Intensity Ratios (SIR) of liver over muscle (L/M), pancreas over muscle (P/M), spleen over muscle (S/M), vertebral bone marrow over muscle (VBM/M) on T1- TSE , T1-GRE , PD-GRE and T2*-GRE MR images, as well as R2 Relaxation rate values (R2=1/T2) of the liver, pancreas, spleen and VBM were calculated in seven multiple transfused patients with Fanconi anemia (n=3), severe aplastic anemia (n=1) , Diamond Blackfan (n=2) and CDA-II (n=1). The same MRI protocol was applied in ten healthy controls and MRI measurements of patients were compared to those of healthy controls. The study was conducted with the approval of the hospital ethics committee, and informed consent was obtained from all of the human subjects. Statistical significance was set at P<0.05. *Results.* R2 calculations, PD and T2*-GRE sequences were the most sensitive MR techniques in tissue iron overload detection : 6 patients were shown to have liver siderosis, 5 pancreatic , 4 splenic siderosis and 6 patients VBM siderosis, as implied by reduced L/M, P/M, S/M and VBM/M ratios on PD-and T2* weighted GRE images and increased respective R2 values, compared to measurements obtained by healthy controls. Despite the limited number of patients, statistically significant correlations were revealed between a) ferritin and L/M on T1-weighted TSE sequence (r=0.85, P<0.04) and PD-weighted GRE sequence (r=0.89, P<0.03) b) ferritin and P/M on T1-weighted TSE sequence (r=0.88, P<0.03) and PD- weighted GRE sequence (r=0.96, P<0.02) c) L/M and P/M on T1-weighted TSE sequence (r=0.81, P<0.03) and d) S/M and VBM/M on PD-weighted GRE sequence (r=0.94, P<0.001). MR measurements of any tissue obtained on T1-GRE and T2*-GRE weighted sequences and R2 calculations did not assign statistically significant correlations with serum ferritin. *Summary/Conclusions.* An intergraded MR imaging protocol can be employed for non invasive assessment of tissue iron overload in patients with transfusional iron overload in rare congenital anemias, other than hemoglobinopathies. Signal intensity ratios on PD-GRE weighted sequence were the most sensitive and accurate measurements for estimation of tissue iron in those patients. Statistically significant correlations between the degree of hepatic and pancreatic siderosis and between VBM and splenic siderosis were found.

1868

A STUDY OF PLATELET INDICES IN PATIENTS WITH IRON DEFICIENCY ANEMIA

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Background. Iron deficiency anemia is the most common hematological disorder in the community. Several changes concerning platelets have been found in this condition, which may connect iron metabolism to platelet indices. IPF is a novel platelet index which raises in several disease states where reticulated platelets are released in peripheral circulation, due to increased bone marrow activity. *Aims.* The aim of this study was to seek correlations among classic and novel platelet indices, as well as with parameters concerning iron metabolism, in patients with iron deficiency anemia. *Methods.* We retrospectively studied 41 patients (26 female and 15 male) with iron deficiency anemia. Platelet (PLT) count, mean platelet volume (MPV), platelet distribution width (PDW), plateletcrit (PCT), and immature platelet fraction (IPF), as well as iron levels, ferritin levels and total iron binding capacity (TIBC) were determined for these patients. Hematological parameters were assessed by SYSMEX XE2100 hematological analyzer, while biochemical parameters were assessed by MODULAR ANALYTICS P800 analyzer. Pearson's correlation coefficient was used for correlations among parameters. The significance level was defined as P<0.05. *Results.* Platelet count was statistically significantly positively correlated with TIBC (r=0,629, P=0,007). Plateletcrit was also positively correlated with TIBC (r=0,648, P=0,012). Significant correlations among platelet indices were also of some interest. Platelet count was negatively correlated with IPF (r=-0,451, P=0,003). Novel IPF index was positively correlated with MPV (r=0,749, P<0,001) and PDW (r=0,755, P<0,001). *Conclusions.* In our study, an increase in platelet number and plateletcrit was correlated with an increase in total iron binding capacity, which reflects a decrease in blood iron levels. Theoretically, this observation could be attributed to increased erythropoiesis (and erythropoietin levels). Moreover, the compensatory increase in platelet number is not followed by an increase in immature platelet forms in peripheral blood. This might reflect a less acute bone marrow response, due to the chronic nature of the disorder, with few immature large reticulated platelets released in peripheral blood.

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OPTIMAL RESPONSE TO ALEMTUZUMAB IN A CASE OF IDIOPATHIC OF PURE RED CELL APLASIA

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Pure red cell aplasia (PRCA) is an uncommon hemopathy, often related to thymoma, autoimmune diseases or lymphoproliferative disorders, whereas almost 50% remain as idiopathic. Usual therapies consist of glucocorticoids, inmmunosupressors or the adequate treatment for the primary disease whenever is known. We present the case of a patient in which the response to alemtuzumab was optimal and complete, after the failure of at least 4 different therapies. *Case.* 58 yr.-old male, firstly studied for macrocytic anemia. He stated with transfusions in 2006, when the diagnosis of PRCA was made (bone marrow exam also showed significant dysplastic features). In the initial survey, no evidence of Parvovirus, thymoma, autoimmunity or lymphoproliferative disease was ascertained; allopurinol was interrupted witout any change in the evolution. Bone marrow genetics was 46-XY. The unique anomaly was the presence of an inverted CD4/CD8 T-lymphocytes ratio, without abnormal populations (flow cytometry) and with controversial results with regard to the presence of clonality in receptor T gene (TCR). Therapies: prednisone 1 mg/kg, effective but he response was clearly dose-dependent and the patient developed significant side-effects (myopathy, osteoporosis); the addition of chlorambucil was not useful to allow the tapering of the prednisone dose, as it was not the replacement of prednisone by high-dose intermittent dexamethasone; IV immunoglobulins were also inefficacious; since [Epo] levels were <200 mUi/mL, rh-EPO started, 30,000 UI/wk, which proved to be successful (the patient's hemoglobin was around 11-12 gr/dL for more than a year with only 10 mg/48 h. of prednisone). In January-2009, he started transfusions again; a new complete workup was performed, confirming the diagnosis of PRCA; karyotype was 46-XY and no evidence of B or T-cell clonality was found. Successive therapies were regarded as failed:darbepoietin plus prednisone 1'5 mg/kg, cyclosporin (withheld due to toxicity), low-dose weekly methotrexate. In Sept.-09, he needed 2-3 blood units every 7-14 days to stay aorund 8 gr/dL of Hgb, and biochemical signs of iron overload were present, despite the early start of deferasirox. Alemtuzumab was approved by compassionate use in Nov.-09. The dose was the same as for chonic lymphocytic leukemia, and with the same prophylaxis (valacyclovir, cotrimoxazol) The reponse was evident since the second week of therapy, with an increase in [Hgb] of more than 3 gr/dL and normalization by the fourth. No complications appeared, except the expected lymphopenia and neutropenia grade 3. Once the optimal [Hgb] was reached, we began a progressive decrease of the dose, to a final 30 mg/wk). After the scheduled 12-week tretament, the reponse is considered complete. *Conclusion.* The evolution of the case shows the potential utility of alemtuzumab in idiopathic PRCA, whereas the exact dosage and the possibility of a maintenance therapy is still uncertain. This use of alemtuzumab is not included in the recommendations of the Prescribing Information document approved for the drug.

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MICROCYTIC ANAEMIAS IN PEDIATRICS: HOW MANY GENETICS?

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Background. Microcytic anaemias in pediatrics are very frequent so to be considered the most common in childhood, generally secondary to iron deficiency. Thalassemias are extimated to involve from 6 to 10 % of the Campania's (Italy) population. *Aims.* Aim of our investigation is to demonstrate that most of microcytemias are genetics (α, β ,δβ thalassemia) (thal). *Methods.* We analyzed 20 children in 2009 (14F 11M, ranged 6months-16yrs, mean age 6.5 yrs) with microcytic anaemia and/or isolated microcytemia. Once we excluded the most common causes of microcytemia and/or failure of treatment with oral iron patients underwent screening for genetics anemias , using Haemoglobin (Hb) phoresis (BIO-RAD VARIANT I KIT). When Hb phoresis shown abnormalities in the phoresis distribution, diagnostic with molecular biology to β and/or δβ thal (Bio-Rad mDx®BeTha Gene 1) was made. When no abnormal Hb came out from phoresis, we investigated by molecular biology to α thalassemias (Kit Bio-Rad mDx® Alpha Gene 2). *Results.* 10/20 evaluated children were affected by non genetic anemias, 10/20 underwent molecular biology. 3/10 had β thal trait; 2/10 δβ thal trait; 3/10i α thal trait; 2/10 no trait was shown (see table below). 1 patient had all the characteristics of a β thal trait, but no mutation was discovered, maybe because not in our panel (rare mutation?) *Summary/Conclusion.* Even if should not be considered a fully epidemiological study because of the few number of patients our investigation, using a rapid and low cost method (the median time for a complete response is about 72 hours, also considering the kit maintenance) shows an incidence of about 40% of genetic anaemias in the analysed sample of the Campania's population and it seems to be much more frequent than previously reported. The high incidence of α thal trait seems to indicate that we have to screen patient for this genetical defect, when in presence of inexplicable microcytic anaemias and /or isolated microcytemia. Further studies are obviously necessary and it should be useful to screen up ,using this kind of methodics, children of primary and secondary school of Campania to introduce a fully epidemiological investigation.

Table.

AGE	RBC	Hb	HTC	MCV	RDW	Hb A_0	Hb F	Hb A_2	Molecular Biology
2 yrs	5.91	11.3	43.4	66.4	18.1	88.6	8.6	2.8	δβ ?
1 yrs	5.41	12.2	38.2	68.5	18.8	78.5	19.2	2.3	δβ
3 yrs	6.11	11.1	33.0	54.0	18.6	92.4	2.3	5.3	β(CD39)
4 yrs	5.18	13.0	40.8	78.8	15.3	96.0	//	4.0	β(CD39
8 yrs	4.38	12.7	39.6	77.6	15.7	97.0	//	3.0	//
4 yrs	5.18	13.5	38.2	72.9	18.3	97.4	//	2.6	//
7 yrs	5.36	11.1	33.2	61.9	18.2	94.3	//	5.7	??????
2 yrs	5.32	11,6	33.8	63.8	20.0	96,8	//	3.2	α.(4,2/20,5)
16 yrs	5.06	8.30	27.3	54.0	19.2	94.9	3.0	2.1	α.(3,7/20,5)
3 yrs	5.09	10.3	20.2	59.0	18.8	97,3	//	2,7	α.3,7/m.

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SCREENING OF HEMOGLOBINOPATHIES IN PREGNANT WOMEN IN GRAN CANARIA

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Structural hemoglobinopathies are a group of diseases that constitute a major public health problem in the world, with an incidence that has increased in recent years in our country due to migration movements. There is a controversy about screening for this disease universally or only in high prevalence popultations. *Patients, materials and methods.* 240 consecutive peripheral blood samples in EDTA-K3 of pregnant women were analyzed in the north area of Gran Canaria. We performed high-resolution liquid chromatography (HPLC) for detection and quantification of abnormal hemoglobins. Data from hemogram and biochemistry of these patients were reviewed. Informed consent was obtained *Results.* We found 19.16% of microcytosis. One of them was a known alpha thalassemia patient and two had increased fetal hemoglobin.The prevalence of heterozygotes for hemoglobin S was 1.66%. We did not find any other structural hemoglobinopathy or any patient with sickle cell anemia. *Conclusions.* Our results show a high prevalence of heterozygotes for S hemoglobin compared to previous studies, probably related to an increment in migration movements.

1872

LEVELS OF RETICULOCYTE INDICES AND RETICULOCYTE HEMOGLOBIN CONTENT IN HETEROZYGOUS β-THALASSEMIA AND IN IRON DEFICIENCY ANEMIA

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Background. Reticulocyte indices, IRF (Immature Reticulocyte Fraction), LFR (Low Fluorescence Reticulocytes), MFR (Medium Fluorescence Reticulocytes), HFR (High Fluorescence Reticulocytes), and mainly the index of hemoglobinisation of reticulocytes (, Reticulocyte hemoglobin content) are new parameters in blood analysis. The Ret-he, which directly reflects the recent hemoglobin synthesis in bone marrow precursors, has been suggested as a useful hematologic marker not only for early diagnosis but also for the monitoring of iron deficiency anemia. *Aim.* In this study, reticulocyte indices and Ret-he compared and evaluated in heterozygous β-thalassemia and in iron deficiency anemia. *Methods.* A retrospective study of two groups, group A: 100 patients with heterozygous β-thalassemia (57 males and 43 females), and group B: 30 patients (10 males and 20 females) with iron deficiency anemia, in which the indices IRF, LFR, MFR, HFR, Ret-he, as well as iron and ferritin were measured. The measurement of reticulocyte parameters were generated by the Sysmex XE 2100, and biochemical concentrations were determined in MODULAR ANALYTICS P 800. *Results.* Ferritin and iron concentrations were found significantly increased in group A in comparison with group B (78.29±44.7 _g/dL *vs.* 12.3±2.12 μg/dL, P<0.001, and 167.42±166.39 ng /mL *vs.* 65.59±12.18 ng/dL, P<0.001, respectively). The levels of Ret-he and HFR were significantly lower in group A compared to group B (20.73±2.84 pg *vs.* 23.8±3.63 pg, and 3.84±3.7% *vs.* 6.99±6.8%, respectively). In group A there was a positive correlation between HFR and ferritin (r = 0.317, P<0.05). The mean values of IRF, LFR and _FR showed no differences between the two groups. *Conclusions.* Ret-he and HFR could be included among red blood indices, and biochemical parameters that are used for differential diagnosis between heterozygous β-thalassemia and iron deficiency anemia.

1873

CONGENITAL NON-SPHEROCYTIC HEMOLYTIC ANEMIA DUE TO ERYTHROENZYME DEFICIENCIES IN VENEZUELAN PATIENTS

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Erithroenzyme deficiency can produce congenital chronic non spherocytic hemolytic anemia (CNHA). The object of this paper is to present the study of patients with CNHA, the biochemical characterization of their RBC enzymes, hemolysis mechanisms *in vitro* and enzymes deficiencies in different Venezuelan population to get the origen of the deficiency. *Materials and methods.* 2393 patients with CHA were studied. G-6-PD and Pyruvate kinase deficiency were studied in 450 people from different regions. Studies done: medical history, hematologies, chemistries, Hemoglobin electrophoresis, globular fragility test, G-6-PD and glycolytic enzymes determinations, G-6-PD electrophoresis, enzyme kinetic, thermo stability test, phagocytosis, intracellular Na-K- Ca, ATP and its changes with CNK The PK was studied in Basenji dogs with the deficiency and its PK was compared with the human PK enzyme. *Results.* From 2393 patients with CHA or jaundice studied, 1511 had hemoglobinopathies, 432 thalassemias, 342 enzyme deficiencies , 108 membrane defects. 271 G-6-PD deficiencies were found (145 hemicygotes,124 heterocygotes) , 42 PK, 3 hexokinase, 3 methahemoglobin reductase, 1 galactose 1 uridil transferase, 6 glutathione reductase deficiencies. Variants of G-6-PD were detected Class I: 4,9% with chronic hemolysis : G-6-PD activity : 7,5-13%, Km G-6-P: 133-200 (NV: 50-70 μM), Km NADP: 3,8-4,4(NV: 2,9 -4,4 μM), instable to the heat, G-6-PD electrophoresis mobility type A(202G-A/376A-G). Class II in 45,7% of the patients with G-6-PD activity : 0-10%, Km G-6-P: 31-200 μM, Km NADP: 2,2-4,4. Electrophoresis type B. Some of these patients had favism. Class III: 49,6% of the patients with G-6-PD activity: 11-58%, Km G-6-P: 40-73, Km NADP 1,4-2,9 μM. Electrophoresis type A and B. Na- K y Ca RBC of G-6-PD deficients were normal. 3 families with G-6-PD deficiency had Chronic Granulomatous Disease. G-6-PD deficiency was found only in Venezuelan mixed population with Caucasians and blacks people. Venezuelan indians did not have the G-6-PD neither PK deficiency. 42 PK deficients were detected (Hb :8,24+/1,24 g/dL; Hto: 28,3+/-2,9;%, retic 25,10%+/-17,21).These RBC incubated with CNK showed K and ATP decreased and Ca increased. 3 families with severe hemolysis, PK deficiency, and ulcers in legs had a biochemical mutant of PK with low KM for PEP 0,75+/- 0.5 μM (NV: 1,15+/0.1) no allosteric similar to PK of Basenji dogs. 4 families had hexokinase deficiency. A mutant Hx with severe hemolysis and ulcers in legs were characterized Conclusions the incidence of G-6-PD and glycolytic enzymes that produces hemolytic anemia and jaundice in Venezuela is presented. La deficiency of G-6-D was inherited from caucasic and black inmigrants. Three classes of G-6-PD biochemical variants, a PK and a hexokinase mutant that produces severe hemolysis and leg ulcer were detected. When PK deficient RBC were incubated with CNK loose K, ATP and increase Ca. The Ca open the K canals and water is also loose as a result the RBC suffer dehydration and probably hemolysis will occur due to phagocytosis. This could be one of the mechanisms to explain hemolysis

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SERUM HEPCIDIN LEVELS IN SUBTYPES OF ANEMIA WITH DISTURBED IRON METABOLISM

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Hepcidin is an 25 amino acid cystine rich peptide, synthesized predominantly by hepatocytes. It was suggested that hepcidin act as a hormone regulating iron content in human body through binding to ferroprotein. The aim of this study was to assess serum hepcidin level in 3 category of patients including; iron deficiency anemia (IDA) (n= 12, age range from 3-9 years); β thalassemia major (n=15, age range from 3- 8.5 years); Hodgkin disease with B symptoms (n = 11, age range 27-48 years); and normal healthy control (n=10, age range 2.0- 8.0 years). Serum hepcidin levels was assayed by ELISA. Hepcidin serum levels was significantly elevated in β thalassemia group and HD group as compared to control (P<0.01 for both). In contrast serum hepcidin level was significantly decreased in IDA group as compared to control level (P<0.05). Serum ferritin level was inversely correlated with hepcidin level in IDA group (r=0.623, P<0.01); but significantly positively correlated in β thalassemia major group and HD group (r = 0.52, P<0.01, r=481 , P<0.05 respectively). These findings suggest that serum hepcidin level could discriminate between simple iron deficiency anemia and other anemia subtypes with disturbed iron metabolism.

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GAUCHER DISEASE AS A CAUSE OF ANEMIA AND THROMBOCYTOPENIA

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Background. Many diseases that are not primarily hematological, as infectious, immunological, neoplastic and metabolic problems present with hematological findings, such as cytopenias, and so the hematologist is often in the first line in their diagnostic evaluation. Gaucher disease (GD) is an inherited deficiency of the enzyme glucocerebrosidase. The underlying defect leads to deposition of glucocerebroside in cells of the macrophage-monocyte system, with excessive accumulation in the bone marrow, spleen, bone and other organs. Cytopenia is an almost universal finding in untreated Gaucher disease. Anemia, thrombocytopenia and to a lesser extent leucopenia, may be observed simultaneously or independently. Other signs and symptoms include splenomegaly or hepatosplenomegaly and variable bone involvement. Malignant hematological diseases can also be associated with GD. Some patients present in childhood with virtually all the complications of GD, whereas others remain asymptomatic until later in life. Although earlier splenectomy was carried out due to functional hypersplenism and the mechanical effects of the enlarged spleen, Enzyme Replacement Therapy (ERT) with imiglucerase is currently available. *Aims.* to present the hematological features at presentation of type 1 GD and resolution after ERT. *Methods.* case report of three patients with GD initially admitted for evaluation in a Pediatric Hematology Unit. *Results.* Patient 1 presented at the age of eight with recurrent and severe bone pain. These episodes were mistaken for recurrent osteomyelitis. At the age of twelve massive splenomegaly and aggravation of anemia and thrombocytopenia were noticed and the patient underwent bone marrow examination that revealed Gaucher cells. Patient 2 was first evaluated at the age of six for fever, anemia, thrombocytopenia and hepatosplenomegaly. Multiple infectious and autoimmune investigations were carried out and proved to be negative. Due to persistent enlargement of liver and spleen and cytopenia a bone marrow aspi-

rate was performed and also showed Gaucher cells. Patient 3 was a four-year-old previously healthy boy when splenomegaly and mild thrombocytopenia were casually noticed. GD was also suspected and investigations for this disorder performed. All patients had low glucocerebrosidase activity in leucocytes and fibroblasts and increased plasma chitotriosidase and tartrate resistant acid phosphatase (TRAP). Magnetic resonance imaging of patient 3 showed bone abnormalities suggestive of Gaucher disease. ERT resulted in total regression of symptoms and hepatosplenomegaly, normalization of hematological parameters and progressive improvement of quitotriosidase and TRAP in all patients. *Summary/Conclusions.* We remember GD as a cause of anemia and thrombocytopenia associated with splenomegaly or hepatosplenomegaly. Bone involvement may be severe as in patient 1 or still asymptomatic as in patient 3. An adequate evaluation and diagnosis are the hallmark of success in the management, as ERT is available and highly effective in this otherwise progressive and debilitating disorder.

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MEGALOBLASTIC ANEMIA AND COBALAMIN DEFICIENCY: THE IMPORTANCE OF AN URINE SAMPLE

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Background. The Imerslund-Grasbeck Syndrome is a rare autosomal recessive disorder characterized by selective malabsorption of Vitamin B12 and asymptomatic proteinuria. The clinical manifestations appear in early childhood, usually within the first two years of life. Anemia, failure to thrive, recurrent infections and/or neurological symptoms (ie: spasticity, ataxia and cerebral atrophy) are its most common presentations. The treatment consists of Vitamin B12 administration, which usually leads to complete remission of symptoms. Proteinuria usually persists in adults but with no impact in quality of life and long-term survival, even in selected cases with altered renal function. *Aims.* to describe the importance of an urine screen in the differential diagnosis of childhood megaloblastic anemia. *Methods.* case report of a patient with pancytopenia and macrocytosis admitted for evaluation in a Pediatric Hematology Unit. *Results.* We present a previously healthy 15-month-old female child with an unremarkeable family or personal history (ie: normal growth and development and no history of recurrent infections or neurological symptoms). During an upper respiratory tract infection, icteric sclerae and progressive pallor of the skin and mucous membranes were noted. The blood test results revealed pancytopenia with macrocytosis and neutrophil hypersegmentation, accompanied by a low serum vitamin B12 level (85.7 pg/mL, normal range: 193 to 982 pg/mL). With the 12-hour proteinuria level of 38 mg/m2/h, a diagnosis of Immerslund - Grasbeck Syndrome was reached, and therapy with subcutaneous cyanocobalamin was initiated, resulting in normalization of hematologic parameters. Currently, at 7 years of age, she maintains excellent growth and development. Both parents present a mild Vitamin B12 deficit. *Conclusion.* The detection of a megaloblastic anemia in childhood should always be followed by a proteinuria screen, which enables the timely and simple treatment of a highly disabling disease.

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EVALUATION OF NT-PROBNP AND HOMOCYSTEINE LEVES IN PATIENTS WITH HEMOGLOBINOPATHIES

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Background. Despite progress in chelation therapy, heart failure is still the main cause of death in patients with hemoglobinopathies. Hyperomocysteinemia is an independent risk factor in pathogenesis of thromboembolic episodes, Early cardiovascular involvement in thallasemic patients without cardiac symptoms has not been adequately investigated.*Aim.*To assess NT-proBNP and total homocysteine (tHcy) serum levels in patients with hemoglobinopathies. *Methods.* We studied 108 patients (mean age: 39,32+19,66 years) with β-thalassemia major (β-T.M), β- thalassaemia intermedia (β-T.INT) and sickle cell anemia (SCA) divided in three groups. A control group (CG) of 40 age-matched healthy subjects was studied simultaneously and costituded a fourth group. Serum levels of NT-proBNP were determined with electrochemiluminescence immunoassay and homocysteine (tHcy) serum levels were determined using Fluorescence Polarization Immunoassay (FPIA).

Results. Our results are present in the Table 1. Serum level of NT-proBNP was significantly higher in patients with β- thalassaemia major (153,55+76,77 pg/mL *vs.* 50,12±25,06 pg/mL, P<0,01),in patients with β-thalassaemia intermedia (297,75+148,85 pg/mL *vs.* 50,12±25,06 pg/mL, P<0,05) and in patients with sickle cell anemia (151,50+75,75 pg/mL *vs.* 50,12±25,06 pg/mL, P<0,01) compared to the healthy group. It is remarkable that mean value of NT-proBNP in patients with β- thalassaemia intermedia is higher than the other groups.It is also interesting that NT-proBNP serum levels increase simultaneously with patients age.Homocysteine serum levels don't increase significantly in patients groups. No correlation was observed between NT-proBNP and homocysteine. *Conclusions.*This is one of few studies assessing NT-proBNP levels with hemoglobinopathies. The most important finding of the present study is the increase of NT-proBNP in all patients compared with normal controls. Complementary therapy with folic acid and B6 in these patients probably keep low the serum levels of homocysteine.

Table 1. Mean values (±SD) of NT -proBNP and tHcy of patients and control group (CG).

GROUP	N	NT-propBNP pg/mL	tHcy µmol/L
CG	40	50,12±25,06	7,89+3,94
β-T.M	54	153,55+76,77*	7,37+3,68
β-T.INT	24	297,75+148,85**	8,95+4,47
SCA	30	151,50+75,75***	8,57+4,27

*p<0,001 **p<0,005 ***p<0,001

1878

COMPARISON BETWEEN IRON SUCROSE AND IRON SUCROSE SIMILAR PREPARATIONS ON OXIDATIVE AND NITROSATIVE STRESS IN THE RAT CENTRAL NERVOUS SYSTEM

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Background. Intravenous (IV) iron is an efficient treatment for iron deficiency. Nevertheless, iron is a highly reactive metal and may cause a variable degree of oxidative stress and tissue inflammation in central nervous system (CNS) structures. In some indications iron may be used on a chronic basis and consequently the awareness of its potential CNS toxicity is of importance. *Aims.* This study evaluates possible differences on oxidative stress, nitrosative stress and inflammatory response in brain tissue between the original iron sucrose (IS) and iron sucrose similar (ISS) preparations from different countries in normal rats.

Table 1.

Mean ± SD	G1 (n=7)	G2 (n=7)	G3 (n=7)	G4 (n=7)	G5 (n=7)
Hb (g/dL)	16.4 ± 0.4	16.1 ± 0.4	16.1 ± 0.3	16.2 ± 0.4	15.9 ± 0.3
Serum iron (µg/dL)	371.3 ± 17.7**	431.4 ± 14.1	426.4 ± 10.6	455.0 ± 21.9	317.1 ± 14.8*
TSAT (%)	68.5 ± 5.7**	85.0 ± 3.5	82.0 ± 6.4	83.1 ± 7.1	44.4 ± 4.9*
TBARS (nmol MDA/g prot.)	217.8 ± 18.8**	309.1 ± 20.6	322.7 ± 21.1	315.4 ± 25.0	191.2 ± 21.5**
GSH/GSSG ratio	6.0 ± 1.2**	3.5 ± 1.2	3.8 ± 1.3	3.9 ± 0.7	6.7 ± 1.1**
Nitrotyrosine (+cells/area)	2.1 ± 1.4**	6.7 ± 2.1	6.3 ± 1.4	6.1 ± 2.1	1.0 ± 0.7**
TNF-α (+cells/area)	3.6 ± 0.8**	10.9 ± 3.5	9.3 ± 1.4	9.0 ± 2.1	2.0 ± 0.7**
IL-6 (+cells/area)	4.2 ± 1.8**	9.8 ± 2.5	9.4 ± 2.7	10.1 ± 2.8	1.4 ± 0.6**
HSP70 (+cells/area)	5.5 ± 2.3**	11.2 ± 2.9	13.0 ± 3.5	11.5 ± 1.9	2.7 ± 1.2**
Caspase-3 (+cells/area)	0.7 ± 0.4**	4.1 ± 1.0	3.5 ± 0.9	3.8 ± 0.6	0.4 ± 0.5**
* p<0.01 vs all groups; ** p<0.01 vs G2, G3 & G4					

Methods. Five groups of Sprague Dawley rats: G1 (IS), G2 (ISS Portugal), G3 (ISS Colombia), G4 (ISS Argentina), G5 (Control saline solution) were compared. G1, G2, G3 and G4 were treated with a weekly IV dose (40 mg iron/kg bw) of the corresponding iron compound, and G5 with normal saline solution at 0, 7, 14, 21 and 28 days. Animals were killed after the last IV dose (Day 29). In brain homogenates, thiobarbituric acid reactive substances (TBARS) and GSH/GSSG ratio were evaluated.

Immunohistochemistry assessments using antibodies against nitrotyrosine, interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), heat shock protein-70 (HSP70) and caspase-3 were also performed. *Results.* At the end of the study (4 weeks), hemoglobin (Hb) did not significantly differ between groups. However, serum iron, transferrin saturation (TSAT), as well as markers of oxidative stress, nitrosative stress, inflammation and apoptosis (caspase-3) showed significant (P<0.01) differences between groups. Results at the end of the study are illustrated in Table 1. *Summary/Conclusions.* These findings demonstrate that the studied ISSs lead to substantial oxidative and nitrosative stress, increased inflammatory response and apoptosis in the normal rat brain *vs.* original iron sucrose. A possible explanation for these observations may be that labile iron is increased in ISSs due to low stability of the iron complex.

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A NOVEL MUTATION OF THE ERYTHROID-SPECIFIC AMINOLEVULINATE SYNTHASE 2 GENE IN A PATIENT WITH PYRIDOXINE RESPONSIVE SIDEROBLASTIC ANEMIA AND DEFERASIROX RESPONSIVE HEMOCHROMATOSIS

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Background. Sideroblastic anemias are a heterogeneous group of acquired and inherited bone marrow disorders characterized by mitochondrial iron overload in precursors of red blood cells. Ring sideroblast is the characteristic finding of all sideroblastic anemias. Pathologic iron deposition and anemia are the most common problem in patients with sideroblastic anemia. Hereditary sideroblastic anemias are caused by defects in genes present of X chromosome, otosomal genes and mitochondrial genes. *Case report.* A-14 year-old man admitted to our clinic with weakness and paleness since one month. He has hepatosplenomegaly. Blood tests and peripheral blood smear showed severe hypochromic, microcytic anemia. There is ringed sideroblasts without dysplastic hematopoiesis in bone marrow cytology. Liver tests were normal. Serum ferritin level was high. Liver biopsy showed heavy parenchymal iron deposition and grade-III fibrosis. Screening for HFE gene mutations was negative. Magnetic resonance T2 star imaging demonstrated that severe iron accumulation in liver and heart. ALAS2 gene screening showed that novel mutation in exon 7 (Gly390Gly, c.1170, C-T). Eventually, he was diagnosed as sideroblastic anemia and hemochromatosis. The patient's anemia improved with pyridoxine. Liver and hearth iron loading detected by T2 star MRI and serum ferritin level were obviously improved by deferasirox treatment. *Conclusion.* These findings suggest that the Gly390Gly in ALAS2 mutation is a novel mutation leading to sideroblastic anemia and hemochromatosis, without hereditary hemochromatosis gene mutations. This mutation can cause sideroblastic anemia that clinically pyridoxine-responsive. Deferasirox may be an effective agent for reduce hepatic and cardiac iron overload in this condition.

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RECORDING OF CHANGES IN HEMOGLOBIN, HEMATOCRIT, FE AND TIBC IN PATIENTS WITH MALIGNACY AT THE DURATION OF CHEMOTHERAPY

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Background - Aim. The changes in hematocrit, haemoglobin and iron values as well as iron-binding ability were investigated in the neoplasmatic patients during chemotherapy. *Material - Method.* 22 patients were examined (9 men and 13 women): 7 were suffered from breast cancer, 5 from lung cancer, 4 from thick intestine cancer, 3 from prostate cancer and 3 with various other type of cancers. 1st sample of blood was received before the administration of chemotherapeutic schedule. 2nd and 3rd blood sample were received 3rd and 7th day after the administration, respectively. *Results.* The percentage increase of iron values average between 1st and 2nd sample was 215%, while in another case this increase was sixfold higher. On the contrary, the higher reduction between second and third sample was 98%, while the average value reduced at 51%. The fluctuation in TIBC was lower, (changes <20%), while almost stable remained the values of hematocrit and hemoglobulin values in the various measurements. *Conclusions.* 1) The iron values and the total iron-binding ability are very high the 3rd day during chemotherapy, while the measurements return in their normal range afterwards the 7th day. 2) Reversely, the hematocrit and hemoglobulin values were not significantly influenced. 3) The simultaneous examination of bone marrow during the chemotherapy would potentially help in the explanation of our observations. Moreover, our findings should be confirmed with further studies.

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REDUCTION OF INVASIVE FUNGAL INFECTION (IFI) IN PATIENTS WITH ACUTE MYELOID LEUKEMIA (AML) UNDERGOIG INDUCTION CHEMOTHERAPY WITH POSACONAZOLE FOR ANTIFUNGAL PROPHYLAXIS

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Introduction. Patients with Acute Myeloid Leukemia (AML) undergoing chemotherapy are at high risk for bacterial and fungal infections, particularly during the induction phase. Anti bacterial and anti fungal prophylaxis with fluoroquinolones and triazoles are used to prevent infectious complications. Recently posaconazole, a "second generation"triazole, with greater potency and increased activity against yeasts and moulds, has been approved for antifungal prophylaxis in AML patients. *Patients and methods.* The infectious complications occurring during the induction chemotherapy in a series of 24 AML patients who received anti-fungal prophylaxis with posaconazole solution 200 mg tid were compared to an historical series of 81 patients, treated with prophylactic itraconazole oral solution 200 mg bid. In all patients intravenous antifungal therapy with liposomal amphotericin, voriconazole or echinocandins was initiated after no more than 5 days of fever not responding to broad-spectrum empirical antibyotic treatment. The number and type of infections were recorded, as well as the number of days with prophylactic treatment and with intravenous antifungals therapy. *Results.* Patients in the study were older than patient in the historical control cohort (median 52 *vs.* 60 years, P=.07). In the historical cohort a neutropenic fever was observed in 69/81 patients (85.2%), while in the study cohort a neutropenic fever was observed in 23/24 (95.8%).(P=.29).The incidence of Fever of Unknown Origin (FUO) was similar in the stidy group as compared to the historical group, as well the incidence of Clinically Documented Infections (CDI) and Microbiologically Documented Infections (MDI).(Tab1).An Invasive Fungal Infection (IFI) was diagnosed in 9 (13.0%) patients in the hystorical cohort (2 "possible", 3 "probable"and 4 "proven") while no fungal infection was diagnosed in the study group (Table 1).However, an intravenous antifungal therapy was administered to a comparable percentage of patients in the two cohorts: 50% (12 patients) in the study group *vs.* 55.6% (45 patients) in the hystorical group (P=.9). The median of days from the beginning of chemotherapy to the initiation of intravenous fungal therapy was also comparable in the two groups (18.6 days in the study cohort *vs.* 16.0 days in the hystorical cohort; P=.15) as well the median days of intravenous antifungal therapy (10.5 *vs.* 15.4; P=.13). The incidence of deaths during the induction phase was higher in the study group as compared to the hystorical cohort : 4 (16.7%) *vs.* 6 (7.41%) respectively (P=.24). All deaths were related to uncontrolled primary infections during the aplastic phase or breaktrough infections in patients with resistant leukemia and lack of granulocyte recovery. *Conclusions.* The prophylaxis with posaconazole was effective in reducing the incidence of IFI among pts with AML, althrough did not reduce the use of antifungal treatment.

Table 1.

	Study cohort	*(n 24)*	*Hystorical cohort*	*(n 81)*
FUO	9	(39.2%)	27	(39.2%)
CDI	7	(30.4%)	9	(13.0%)
MDI	7	(30.4%)	24	(34.8%)
IFI	0	9	(13.0%)	P=0,04

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HBV AND HCV PHENOMENONS IN PATIENTS WITH HEMATOLOGICAL MALIGNANCIES

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Background. Viral hepatitis B and C are one of the most common cause of severe liver disfunction, including fulminant hepatitis, in patients with hematological malignancies due to reactivation or *de novo* infection. Earlier we postulated that the rate of infectious process caused by viruses hepatitis B (HBV) and hepatitis C (HCV) was extremely high in the hematology department (for HBV from 15% (39/265) to 51% (135/265) and for HCV from 7% (19/265) to 19% (51/265), 14% (37/265) pts had markers of both types of viruses) [14th Congress of EHA, 2009, abstr 0466]. Furthermore, HBV and HCV can cause prolonged bone marrow aplasia in patients receiving chemotherapy or immunosuppressive agents. *Design and methods.* We perfomed a prospective study to assess the outcomes associated with HBV and HCV infections in all patients admitted to hematology department since Feb 2004 till Jun 2006. Median follow-up was 253 days (3-1412). All unselected 265 patients were monitored prospectively by testing of HBsAg, anti-HCV, DNA-HBV, RNA-HCV, anti-HBs, anti-HBc, HBeAg, anti-HBe monthly. Total 7800 biological samples (serum, plasma, peripheral blood cells, spinal fluid, saliva, bone marrow aspirates, bone marrow, liver and spleen biopsies) were collected for HBV and HCV testing. 4000 of them were tested by PCR assays. *Results.* 205 pts (77.5%) of all monitored cases were pts with acute leukemia (AL) and aplastic anemia (AA). 125 (47.0%) were men and 140 (53.0%) were women; their median age was 38 years (15-79). Within the period of follow-up 173 pts (65.7%) were detected to have any positive marker for HBV and 69 pts (26.0%) - for HCV. 57 pts (21.5%) were infected with both HBV and HCV, that constituted 32.8% of those 174 pts infected with HBV and 81.4% of those 70 pts infected with HCV. At time of analysis 152 pts (57.4%) were alive. 113 pts (42.6%) died. 78 (69.0%) of 113 died pts were infected with HBV and HCV and two of them developed fatal fulminant hepatitis. 30 (38.5%) of those 78 pts became positive for DNA-HBV and RNA-HCV by PCR in serum, or bone marrow, or spleen, 3 to 30 days before death. This phenomenon may be explained by rapid destruction of HBV and HCV infected cells by immune attack. But it's more likely that in our cases host immune factors is collapsed and cannot control the infections. We can speculate that at the time of vital stress in order to survive all cells including those harbouring viral factors induce intracellular antiapoptotic mechanisms thus providing untolerable conditions for viral intracellular persistence and viruses leave the cell. This suggestion is supported by the fact that in some patients viral antigens were detected by immunohistochemical methods in bone marrow biopsies without serological and molecular HBV and HCV markers in serum. *Conclusions.* The present study has brightly demostrated that the patients in hematological departments have to be strictly monitored for HBV and HCV by all existed methods in all reasonable biosubstratum, particularly by PCR and immunohistochemical *Methods.* The high incidence of DNA-HBV and RNA-HCV in died patients leads us to a conclusion that these viruses are not the reasons of death but just markers of vital stress. The absence of molecular and serological markers of HBV and HCV does not mean the abcence of these infections in this cohort population.

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DAPTOMYCIN IN THE TREATMENT OF GRAM POSITIVE INFECTIONS IN PATIENTS WITH MALIGNANCIES ONCOHEMATOLOGICAL AND/OR NEUTROPENIC: SPANISH DATA EUCORE REGISTRY

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Background. Given the situation of multidrug resistance and therapeutic failure of traditional antibiotic therapy is necessary to have updated information on efficacy and tolerance of new antibiotics against Gram positive (GP) and Daptomycin (DPM) in the context of special guests immunocompromised, and patients with neoplasms (PN) solid or hematological, with or without neutropenia (NP). *Aims.* Evaluation of national data on efficacy and safety of Dpm in the treatment of various serious infections complicated by GP or PN from a European registry (EUCORE). *Methods.* Retrospective cohort study based on the selection and analysis of the PN included by Spanish researchers in the general database registry EUCORE (free communication experience and results of use of daptomycin in infections proven or highly suspected to be caused by GP) recorded between 2006 and September 2008 in Europe. *Results.* The Spanish contribution to record EUCORE (1127 patients) included 345 patients treated with Dpm, most treatments 'rescue' (290, 84%), a percentage of 35% in critical condition (n = 122) and having> 65 years 44% of them. The 40.32% of infections were caused by S. aureus (14.71% MRSA) and 30.83% for coagulase negative (ECN). Among them were detected 80 PN with cancer (23.19%), 21 of those admitted to the ICU. The type of cancer described in the study was hematologic cancer and solid organ cancer at the start or during the use of Dpm. 51% of them were treated with doses of 6mg/kg/d Dpm, and median days of treatment was 12 days, 14.5 days in cases of Np). GP bacteremia associated with vascular catheter (CV) (19, 24%) or not associated with CV (10, 12%) were the main types of infections in cancer patients. There were 9 infections associated with foreign bodies (11%) and 9 cases of endocarditis (11%), IcPTB 16 cases (20%). In terms of efficacy (removing 10% of evaluable patients) had successful overall therapeutic effectiveness in 77% of the NPs with cancer (83% in bacteremia, endocarditis 78%, 78% in foreign bodies). In the subgroup of PN with cancer to stay in ICU (21) the effective response rate was 81%. In the 14 PN with Np, the favorable response rate was 93% (75% in cases of bacteremia, 100% in the 6 patients with <500 neutrófilos/mm^3). No adverse effects were detected that would force the withdrawal of the DAP, although in one case there was an increase in the number of CPK than 10 times. For the total patients (n = 345) were 36 patient died (10.43%), being just one of them possibly related to the drug and recorded as sudden death. *Conclusions.* Dpm constitutes a very important antibiotic option with good efficacy and safety results in the treatment of serious infections by GP in PN, with or without NP.

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NOVEL INFLUENZA A (H1N1) OUTBREAK IN HEMATOLOGIC MALIGNANCY AND HEMATOPOIETIC STEM CELL TRANSPLANTATION (HCT) PATIENTS

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Background - Aims. We report the clinical course and outcome of novel influenza A (H1N1) outbreak in immunocompromised patients with hematologic malignancy or HCT. All febrile ambulatory and hospitalised patients in our department were checked for H1N1 by PCR. Vaccination was performed to selected patients according to the guidelines. *Methods.* Eight patients with novel influenza (H1N1) infection were confirmed in our department between 22.12.2009 and 04.01.2010. Four patients had acute myeloid leukemia (AML). Three of the AML patients were neutropenic after induction regimens and one due to resistant disease. One patient had common variable immunodeficiency and newly diagnosed large B-cell lymphoma. Three patients had undergone HCT: one patient had extramedullary relapse of AML post HCT and developed symptoms after hematologic recovery, one suffered from severe pulmonary cGvHD 18 months post HCT, and one had CNS relapse 15 months post HCT and was vaccinated against the H1N1 virus. *Results.* Three patients had mild clinical course, presenting with fever, upper respiratory tract symptoms and normal radiological findings. Five out of eight patients had progression to severe lower respiratory tract disease. Clinical signs were fever, cough, tachypnea, rales and abnormal arterial blood gas values. Chest CT findings were nodular infiltrations, air-bronchogramm, ground-glass and tree-in-bud opacities, bronchiolitis and pleural effusions. On day 7, all five patients had severe respiratory distress and three eventually required ICU care. Three of them died. Two developed also gram negative sepsis by *Klebsiella* pneumoniae and *Burkholderia cepacia* and the other one pulmonary aspergillosis. The patient with *Klebsiella* pneumoniae suffered also from severe pulmonary cGvHD. Among the patients who survived from severe disease, two were afebrile by day 30 but unable to keep an adequate pO_2 without oxygen supply. Findings of pulmonary fibrosis were observed in their chest CTs. All patients received oseltamivir 150 mg qd. One of the AML patients had prolonged viral shedding for 22 days. His antiviral treatment was switched from oseltamivir to inhaled zanamivir. *Summary/Conclusions.* The incidence of novel influenza A (H1N1) for hospitalized patients was 5%. The progression to severe lower respiratory tract disease seems to be affected by the patient's

hematological status at the time of infection. The patient who had extramedullary AML and two out of three non-neutropenic patients didn't progress to severe lower respiratory tract disease. The only non-neutropenic patient who developed severe disease had severe refractory pulmonary cGvHD and bacterial infection. Among the five patients with complicated disease, the outcome was strongly associated with primary disease status and cofactors concerning bacterial superinfections and comorbidities. The two patients who eventually survived the severe infection were in CR after recovery from neutropenia. The three deceased patients had additional coinfections, refractory disease (two) and pulmonary cGvHD (one). Post infection pulmonary fibrosis with need of ambulatory oxygen therapy developed in the two patients who survived from severe low respiratory tract disease.

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COMMUNITY ACQUIRED RESPIRATORY VIRAL INFECTIONS IN HOSPITALIZED EGYPTIAN CHILDREN WITH HEMATOLOGICAL MALIGNANCY: PREVALENCE, RISK FACTORS AND CLINICAL OUTCOME

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Background. Intensified chemotherapy increases susceptibility to infections. Respiratory viruses, including respiratory syncytial virus (RSV), parainfluenza virus and Influenza virus A and B are widespread in the community and easily transmitted to cancer patients. *Aim.* to assess the prevalence, risk factors and prognosis of community acquired respiratory viruses in severe lower respiratory tract infections in pediatric cancer patient. *Methods.* Ninety children with cancer admitted in the Children's Hospital, Ain Shams University from March 31st, 2007 until September 30th, 2008 suffering from severe lower respiratory tract infections (LRTIs) were included; their age ranged between 8 months-13 years (mean 6.5±2.8 years), they were 49 males and 41 females. All were subjected to history and clinical examination; investigations included complete blood picture, ESR, CRP, blood culture, plain chest x-ray, computed chest tomography. A nasopharyngeal swab was examined by multiplex PCR for influenza A and B, parainfluenza serotypes 1 and 3 and respiratory syncytial virus. *Results.* 57 patients (63.3%) had hematologic malignancy (47 ALL and 10 AML); and 33 had solid tumours (15 NHL, 3 HD, 8 neuroblastoma, 5 wilms tumour, 2 histiocytosis x). Forty one patients (45%) were in remission and 49 (54%) were in induction/consolidation phase. PCR of nasopharyngeal swabs were positive for viral infection in 34 patients (37.7%): 16 had Influenza A (17%), 3 had Influenza B (3.3%), 9 had Parainfluenza 1 (6.6%), and 5 had Parainfluenza 3 (5.5%), none had RSV . Viral infection was present in 38.5% and 36.3 % of patients with hematological and solid tumors respectively. Wheezy chest was prominent presenting feature of viral LRTI occurring in 52.9% .Bacteria were identified as single cause of LRTI in 16 cases (17.7%), viruses in 25 (27%), fungi in 4 (4.4%) and mixed in 13 cases (14%) [9 mixed viral and bacterial (6.6%), 4 mixed bacterial and fungal infections (4.4%)]. Eighty seven patients (96%) were neutropenic, 31 of them were positive for viral infection (35%). Only 7 patients with viral infection (20.5%) had abnormal CT findings (patchy infiltrates and/or lung collapse). Eight patients (8.8%) were admitted to pediatric ICU; 5 had mixed viral and bacterial infections and 3 had mixed bacterial and fungal infections. Six patients died during the study (6.6%) from infection related sequelae. Bad prognosis including ICU admission and/or death were associated with younger age (<2 years), induction/consolidation phase, signs of malnutrition, exposure to repeated invasive procedures, mixed pathogen etiology ,and presence of neutropenia. By multi-regression analysis, the most predictive factors for bad prognosis were the severity and duration of neutropenia followed by the mixed pathogen etiology. *Conclusions.* Viral etiology should be suspected with incorporation of antiviral therapy in high risk cancer patients as there are no specific signs or symptoms; moreover mixed bacterial and viral infections have the worst prognosis. Radiological findings are non specific and rapid diagnostic viral tests should be routine work up in moderate/severe respiratory tract infections in cancer patients.

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FLOWCYTOMETRIC IMMUNO-PHENOTYPING OF CORD BLOOD MONONUCLEAR CELLS IN SUDANESE NEWBORNS: PATTERNS OF HEALTH AND DISEASE

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Objective. The general goal of this Study is determine the immunophenotypic patterns of cord blood mononuclear cells in a cohort of Sudanese newborns. And use this antigenic phenotype as surrogate markers of health and early infections. *Introduction.* Immunophenotyping of cord blood mononuclear cell is an important tool in the diagnosis and follow up of Newborn with congenital immunodeciencies, HIV infection, or other immune disorders. Infection is still an important cause of neonatal morbidity and mortality, despite the development of broad spectrum antibiotics and advanced life support machines. Serological tests and isolation of microorganisms do not give immediate results and haematological tests that are currently used can be difficult to interpret. Leucocytosis is difficult to interpret due to the wide "normal" range in the newborn (8-32.5×10^3/mL) and an increase. in neutrophil immature forms is subjective, with different morphologists giving variable *Results.* The expression of surface functional antigens on neutrophils, and their upregulation in infection is difficult to interpret due to the effects on these surface antigens by a number of variables such as anticoagulants, type of fixative used and cell separation procedures. In addition, such tests require immediate processing and are time-consuming. *Material and method.* Following institutional ethics approval from ethical committee in instuat of endemic diseases and parental consent, cord blood was collected into K3-EDTA for full blood counts, peripheral blood films examined and immunophenotyping by flow cytometry. The study population divided in to possibly infected (n=99) and non-infected (n=111) depending on the mother's account during pregnancy. *Result.* There was no different in the lymphocyte absolute count between term and preterm infants (p value= 0.2),but with statistically significant difference in absolute lymphocyte between infected and non infected neonates (p value =0.003). There were significant differences in the immunophenotypic patterns of mononuclear cells types in different newborns groups at the time of birth with respect to history of infection (P<0.005). The proportion of CD3$^+$ cells increased with gestational age and with the history infection. In addition, neonates born to mothes with a history of infection had significantly less CD4$^+$ cells and CD4$^+$/CD8$^+$ double-positive cells than neonates with no history of infection. It was also shown that the CD4$^+$/CD8$^+$ cell ratio was increased in neonates with infection. Cells that were reactive to CD4$^+$/CD45RO$^+$ and CD8$^+$/CD45RO$^+$ were more in the infected group compared to those with no history of maternal infection. A strong positive correlation in CD45RO$^+$ cells and a negative correlation with CD45RA$^+$ cells were seen with increased gestational age. In conclusion, T-cells, B-cells and mononuclear cells with CD45 RA & RO reactivity are similar in pre-term and full term babies in the absence of infection. Increased CD45RO$^+$ expression can be a valuable test for screening and monitoring early infections in the newborn. The incorporation of the CD13 (myeloid) marker and early activation markers such as CD69 and CD25 may greatly increase the chance of detection of early infections.

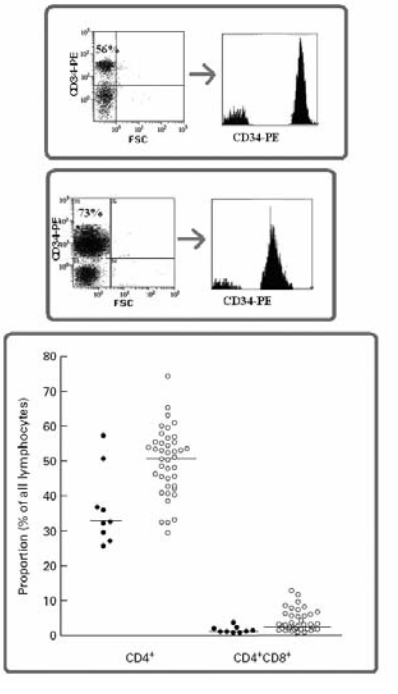

Figure.

Table.

	Term (n=162) Infants	Preterm (n=48) Infants	*P* value
	Mean ± SD	Mean ± SD	
CD3+ Cells (%)	71.9 ±12.2	73.4 ±11.4	0.6
CD3+ Cells ($X10^9$/L)	3.8 ±1.0	4.1 ±2.5	0.4
CD4+ Cells (%)	52.7 ±12.7	54.2 ±13.4	0.6
CD4+ Cells ($X10^9$/L)	2.8 ±1.6	3.0 ±1.7	0.5
CD8+ Cells (%)	19.2 ±3.0	19.1 ±4.8	0.9
CD8+ Cells ($X10^9$/L)	1.00 ±0.6	1.1 ±0.8	0.4
CD45RA+ Cells (%)	62.0 ±14.4	61.9 ±11.2	0.9
CD45RA+ Cells ($X10^9$/L)	3.0 ±1.3	3.4 ±1.8	0.2
CD45RO+ Cells (%)	36.2 ±8.2	35.2 ±8.6	0.6
CD45RO+ Cells ($X10^9$/L)	1.91 ±1.1	2.0 ±1.3	0.7
CD19+ Cells (%)	26.6 ±9.6	23.65 ± 10.79	0.2
CD19+ Cells ($X10^9$/L)	1.33 ±0.8	1.24 ± 0.76	0.6

	Non infected (n=99) Mean	Infected (n=111) Mean	*P* value
CD3+ Cells (%)	64.14 ± 11.45	72.20 ± 12.01	0.000*
CD3+ Cells ($X10^9$/L)	3.11 ± 1.24	3.86 ± 2.17	0.003*
CD4+ Cells (%)	46.09 ± 9.55	52.98 ± 12.77	0.000*
CD4+ Cells ($X10^9$/L)	2.10 ± 0.76	2.84 ± 1.66	0.000*
CD8+ Cells (%)	18.07 ± 4.90	19.16 ± 4.911	0.108
CD8+ Cells ($X10^9$/L)	1.37 ± 1.65	1.03 ± 0.64	0.047*
CD45RA+ Cells (%)	71.27 ± 8.18	61.95 ± 13.73	0.000*
CD45RA+ Cells ($X10^9$/L)	4.05 ± 0.82	3.11 ± 1.40	0.000*
CD45RO+ Cells (%)	23.24 ± 8.67	35.99 ± 8.23	0.000*
CD45RO+ Cells ($X10^9$/L)	1.05 ± 0.47	1.93 ± 1.17	0.000*
CD19+ Cells (%)	31.08 ± 11.36	25.98 ± 9.85	0.001*
CD19+ Cells ($X10^9$/L)	1.39 ± 0.54	1.31 ± 0.77	0.0004*

	Term (n=162) Mean ± SD	Preterm (n=48) Mean ± SD	*P* value
Total White Blood Cells Count	13.95 ± 6.00	14.57 ± 7.20	0.676
Myeloid Cells (%)	49.87 ± 6.53	48.44 ± 6.48	0.351
Myeloid Cells Count ($X10^9$/L)	6.92 ± 3.23	7.08 ± 3.81	0.843
CD14+ Cells (%)	71.86 ± 10.14	73.22 ± 9.32	0.564
CD14+ Cells ($X10^9$/L)	1.37 ± 0.71	1.35 ± 0.76	0.922
CD33+ Cells (%)	51.27 ± 7.11	53.52 ± 7.56	0.185
CD33+ Cells ($X10^9$/L)	4.26 ± 2.02	4.60 ± 2.26	0.486
CD13+ Cells (%)	65.14 ± 7.50	65.83 ± 7.49	0.695
CD13+ Cells ($X10^9$/L)	5.52 ± 2.96	5.85 ± 3.3	0.647

	Non Infected (n=99)	Infected (n=111)	
CD14+ Cells (%)	64.86 ± 15.33	65.28 ± 7.47	0.797
CD14+ Cells ($X10^9$/L)	4.36 ± 1.43	3.59 ± 3.04	0.002*
CD33+ Cells (%)	53.51 ± 12.92	51.74 ± 7.23	0.216
CD33+ Cells ($X10^9$/L)	4.22 ± 2.47	4.33 ± 2.06	0.719
CD13+ Cells (%)	73.00 ± 7.24	72.14 ± 9.95	0.491
CD13+ Cells ($X10^9$/L)	1.06 ± 0.29	1.37 ± 0.72	0.000*

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BACKGROUND, OBJECTIVES, AND METHODOLOGY OF MONITOR-GCSF - A PHARMACO-EPIDEMIOLOGICAL STUDY OF THE MULTI-LEVEL DETERMINANTS, PREDICTORS, AND CLINICAL OUTCOMES OF FEBRILE NEUTROPENIA PROPHYLAXIS WITH BIOSIMILAR FILGRASTIM

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Background. Febrile neutropenia (FN) is a frequent and potentially life-threatening complication in cancer patients undergoing chemotherapy. FN may trigger treatment delays and thus jeopardize the effectiveness of antineoplastic treatment. FN is also associated with increased morbidity, mortality, and health care costs, and decreased quality of life. Biosimilar filgrastim (Zarzio®) has been approved by European authorities. There have been no longitudinal observational effectiveness studies on this agent. We describe the background and methodology of the pan-European MONITOR-GCSF study. *Aims.* Using an integrated framework for post-approval observational studies, MONITOR-GCSF aims to: (1) examine patient- and physician/center-related determinants of response to FN prophylaxis with biosimilar filgrastim; (2) identify patient profiles of vulnerability to poor prophylaxis outcomes; (3) model occurrence of FN breakthrough episodes; (4) identify predictors of non-response; and (5) evaluate the extent to which prophylaxis is in accordance with EORTC guidelines and approved label. *Methods.* MONITOR-GCSF study is an international, prospective, observational, pharmaco-epidemiological study of FN prophylaxis with biosimilar filgrastim in cancer patients undergoing chemotherapy (stage III or IV breast cancer; stage III or IV bladder cancer; stage III or IV non-small cell lung cancer; metastatic prostate cancer; and stage III or IV diffuse large B-cell lymphoma). As of 1 January 2010, this pan-European study has been recruiting at least 1000 patients from a minimum of 75 centers. Patients are followed over a maximum of six chemotherapy cycles. Statistical analyses procedures will consist of: descriptive and associative procedures; variance attribution methods to detect physician class effects; hierarchical linear, logistic, and Poisson modeling of clinical outcomes; Kaplan-Meier time-to-event analysis, Mantel-Cox log-rank or generalized Wilcoxon-Breslow tests, and Cox proportional hazards modeling; and clustering and related data mining techniques. *Results.* Results-reporting will include: baseline center and physician-investigator characteristics; baseline sample characteristics; interim analyses after patients enrolled in the first three months have completed 6 cycles of follow-up (approx. month 9); and 12 (approx. month 21) and 24 (approx. month 33) months later, using Pocock-adjusted levels of statistical significance; similar interim analyses on additional accrual cohorts; and end-of-study analyses. *Summary/Conclusions.* MONITOR-GCSF will provide valuable scientific and clinical information about the patient- and physician-related determinants of FN prophylaxis with biosimilar filgrastim in Europe. It will generate profiles of patients at risk for poor response to FN prophylaxis. New insights in how variability in practice patterns is associated with variability in outcomes will inform clinical practice. By comparing patterns of FN prophylaxis with biosimilar filgrastim to the EORTC guidelines, it will be possible to examine the association between guideline-compliant management of patients and clinical outcomes.

1888

THE MOLECULAR PRESENTATION OF HUMAN PARVOVIRUS B19 INFECTION IN TRANSIENT BONE MARROW SUPPRESSED PATIENTS

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Background. The pathophysiologic etiology which led to transient bone marrow suppression is unknown, complicate, and multifactor. Blood born and bone marrow tropic viruses including human parvovirus B19 infection is one of the possible etiologic agents of this hematopoetic crisis. *Aim.* In this study the hematopoetic presentation of human parvovirus B19 is evaluated for determination of the possible role of this viral infection in pathogenesis of transient bone marrow suppression. *Methods.* In this investigation 27 EDTA treated blood samples where collected from patients with transient bone marrow suppression between years: 2007-2009. The genomic presentation of human parvovirus B19 was determined by an in-house qualitative Nested-PCR protocol. The relationship between B19 infection and the hematologic and biochemical demographic data of all studied patients was analyzed by SPSS version 16. *Result.* The single stranded-DNA of human parvovirus B19 was detected in 7 of 27(26%) transient bone marrow suppressed patients. Also the molecular presentation of Adenovirus was rule out from B19 infected patients. *Conclusion.* Diagnosis of high molecular incidence of human Parvovirus B19 infection in patients with transient born marrow suppression re-enounce the important role of B19 infection in transient hematopoetic crisis and related clinical outcomes.

1889

MICAFUNGIN PROPHYLAXIS IN HEMATOPOIETIC STEM CELL TRANSPLANTATION -- A SINGLE INSTITUTE EXPERIENCE

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Backgroud. Fungal infection is one of the major complications in hematolpoietic stem cell transplantation. In randomised trials we can see the benefit of fluconazole prophylaxis as compared with placebo. Meanwhile, micafungin prophylaxis has the comparative benefitial effect or even better as compared with fluconazole. *Methods.* Between April 2007 and April 2009, 55 patients of hematologic malignancies (AML, MDS, ALL, CML, lymphoma, and myeloma) underwent allogeneic (n=33) or autologous (n=22) peripheral blood stem cell transplantation at our institute. All the transplant patients received micafungin 50mg a day as fungal prophylaxis from day +1 to +30. The preparative regimens include BUCY, TBI/CY, FluBu+/-ATG for AML, MDS, ALL, or CML; BEAM or TBI 2Gy/Flu for lymphoma; melphalan 200 for myeloma. *Results.* There were 12 events occurred which make micafungin prophylaxis shifted to empirical treatment. In these 12 events, there were 2 cases documented to be fungal infection and another 2 cases were highly suspicious fungal pneumonia. The other events ultimately proved to be CMV pneumonia (n=3), engraftment syndrome with idiopathic pneumonia (n=2), pulmonary GVHD or

bronchiolitis obliterans (n=1), recurrent Hodgkin's lymphoma (n=1), and acute lymphoblastic leukemia with liver and lung relapse (n=1). *Conclusions.* The effectiveness of micafungin prophylaxis in our 55 HSCT patients was 78.2% (43 in 55) and the breakthrough infection was 7.27% (4 in 55).

1890

MEDITERRANEAN VISCERAL LEISHMANIASIS: 20 YEARS TREATMENT EXPERIENCE

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Background. Mediterranean visceral Leishmaniasis is endemic disease in some arias in Macedonia. *Aims.* The aim of the study is to present 20 years experience of treatment of with single agent (Nmethyl-glucamine antimonate). *Methods.* During past 20 years (January 1990-january 2010) were diagnosed and treated 48 infants and children, 25 male, the age from 5 months to 12 years. Seven of them were infants. All 48 patients underwent standard clinical examination, biochemical investigation and bone marrow aspiration (positive finding of the parasite in 42 pts). In one patient was done hepatic biopsy and in one biopsy of lymph node. Serological method (Indirect immunofluorescent antibody test (IFAT)) was performed in 7 patients (therapy was applied in 3 pts according only to positive test). All patients were treated with Nmethyl-glucamine antimonate (60 mg/kg BW) i.m., in 14 days cycles and pause of 14 days in between. The cycles were repeated accordingly to the clinical and biochemical data before each cycle. The treatment was stopped when biochemical analyses and bone marrow aspirates were normal. *Results.* In 2 patients the treatment was stopped after first cycle, in 15 patients after 2 cycles, in 30 patients after 3 cycles and in one after 5 cycles (in circumstances of lacking of another effective drug). There were not any toxic side effects of the drug. *Conclusion.* The treatment of Mediterranean visceral Leishmaniasis with Nmethyl-glucamine antimonate in 14 days cycles and pause of 14 days in between is efficient and without toxic effects, even when the cycles are repeated several times, easy for application and not very expensive.

1891

ANALYSIS OF FALSE-POSITIVE RESULTS IN ELISA TEST FOR HIV-1 IN BLOOD DONORS WITH POSITIVE SEROLOGY OR INCONCLUSIVE

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Background. Disposal of blood donors by serological marker for human immunodeficiency virus type I (HIV-1 +2) gains a greater importance for the possible consequences and the clinical severity of infection and the virus becomes important target of preventive measures, including the haemovigilance. Hence the need for additional testing (confirmatory) more sensitive and specific in order to determine the HIV status of the donor. *Aims.* This study aims to determine the frequency and profile of false positive results shown by ELISA, using the Polymerase Chain Reaction (PCR) as confirmatory test. *Methods.* We analyzed 328 samples collected from donors who returned to confirm the serology was positive or indeterminate for HIV-1 at the time of donation. The period of sampling was in April 2008 to March 2009 in HEMOPE - Blood Center Recife. For confirmation of the tests were carried out reactions of nested PCR for 3 regions of the viral genome (GAG, ENV - 2 regions - and POL) from the proviral DNA, making a total of 4 reactions, nested PCR for each sample. For purposes of diagnostic criteria, it was determined that the donor will be told positive for HIV-1 to report a positive result in at least three of the four regions studied. Also included were results of indirect immunofluorescence and Western blot in the study. *Results.* Returned the following results. retest by ELISA, 52 (15.85%) were positive, 141 (42.99%) were inconclusive and 135 (41.16%) were negative. When the samples were analyzed by the reactions of 4 nested PCR for HIV-1, it was found that 282 samples (85.98%) were negative and 46 (14.02%) were positive. The results of the reactions of nested PCR were consistent with the results obtained in indirect immunofluorescence and Western blot. Of the 46 donors confirmed positive by PCR, only 3 (6.52%) were female and 43 male (93.48%). The age where the greatest number of positive individuals was 18 to 30 years (45.65%). *Conclusions.* Whereas the normal window period around 23-30 days, we can consider that these results indicate a high frequency of false-positive reactions in ELISA, and using a molecular technique can eliminate the vast majority of indeterminate results in serological defining better serostatus of the donor. PCR as a diagnostic test can make a transfusion service in reducing risk and damage to health.

1892

HEMATOLOGICAL AND INMUNOLOGICAL MANIFESTATIONS OF HIV-AIDS IN VENEZUELAN PATIENTS

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Background. HIV virus produces immunological and hematological manifestations due to alteration host immune system, complication of secondary infections, malignancies, and as direct effect therapy on hematopoiesis. Our objective was to investigate the frequency and characteristics of 289 Venezuelan AID patients. *Patients and methods.* 322 Patients (164 male and female (nonpregnant) patients, 158 pregnant women) were diagnosed of HIV-AIDS by clinical presentation and tests ($CD4^+$ and $CD8^+$), positive serology and Western blot and hematologies. *Results.* Anemia developed in 106 (65%) patients: 10 with Hb below 10 g/dL, 30 10-12 g/dL, and 66 patients 12-14 g/dL. CD4+ were 304±243 cells/μL, with a CD4/CD8 index 0.36±0.18, viral load 22,730±8,950 RNA copies/mL. 6 required transfusions. Of the anaemic patients, 7 developed mild neutropenia (ANC: 1000-1500/μL); moderate neutropenia (ANC 500-999/μL) was observed in 4 without associated anemia; no severe cases of neutropenia were observed. 10 patients (6.1%) developed thrombocytopenia (50.000-100.000/μL); of these, 3 cases had anaemia. 158 pregnant women were HIV positive (3 first trimester, 52 second and 103 third). Immunological Studies: Third trimester CD3: 1388.94±578.71, CD4: 580.6±312.4, CD8: 840.8±370.7, CD4/CD8: 0.65±0.37; plasma viral load: 15677±35231. Second CD3: 1482.6±619.85, CD4: 427.6±299,576, CD8: 956±593.32, CD4/CD8: 1.4±0.6. Plasma Viral Load: 22061±2790.13. First trimester CD3: 1629±28.26, CD4: 841±2.8, CD8: 646±64.34. Plasma viral load: 3780±4057.4. The CD4 cell count 200-400 in 20.5% of pts, CD4 100-200 in 4.7% pts, CD4 < 100 cells count in 5,1% pts. Hematological and chemical studies third trimester: Hb 11.45±1.3 g/dL, crit: 35.3±4.1 %, WBC: 8443±3187/μL, Platelets: 217477/μL±134.7. Second trimester: Hb 11.6±1.4g/dL, crit: 34.2±3.8%, WBC: 8414±2133/ul, Platelet: 273222±91407/μL Third trimester: Hb 12.9±0.2 g/dL, crit: 39± 0%, WBC: 7600± 500/μL, Platelet: 280000± 50000/μL. The chemistries were normal except one patient with hepatic enzymes high. 152 pregnant HIV positive patients had anemia but two had Hb < 8 g/dL. WBC and Platelets were normal. 25 patients (1.74%) were positive for HIV: 18 NHL (72%) and 7 HL (34.52%). The dominant immunophenotype in the NHL was B cell. 23 patients developed anemia, 2 with Hb 8g/dL; 19 had leucopenia and 2 thrombocytopenia. 4 had Kaposi sarcoma. *Conclusions.* Our series of AIDS patients shows a frequency of anemia of 65%, in a few with mild neutropenia or thrombocytopenia. Isolated neutropenia or thrombocytopenia was uncommon. They had a low index CD4-CD8 with high viral load. 1,5 % of the pregnant women had HIV infection, the majority are in stage I, 9.8% has CD4 <200, 30.7% has anemia and severe anemia only 2 patients The WBC and Platelet count were normal . 92% lymphoma patients developed anemia, 2 with Hb <8 g/dL; 76 % had leucopenia and 2 thrombocytopenia, 4 had Kaposi sarcoma.

1893

THE USE OF DIFFERENT CYTOKINES TO PREDICT THE OUTCOME OF ONCO-HEMATOLOGIC PATIENTS WITH FEBRILE NEUTROPENIA

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Background. Febrile neutropenia is a major complication in onco-hematologic patients. As some patients are at an high risk of contracting life-threatening infections, use of inflammatory markers, besides clinical evaluation, might improve prognostic and aim a more tailored approach. *Aims.* This study aimed to test the association of plasma levels of different inflammatory markers, named CXCL8 (IL-8), CXCL10 (IP-10), tumor necrosis factor alpha (TNFa) and its two soluble receptors, type I and type II (sTNFRI and sTNFRII), CCL2 (MCP-1),CCL3 (MIP-1α), procalcitonin (PCT) and CCL11 (eotaxin) with in-hospital mortality, fever persistency (after 3 days of antimicrobial treatment), bacteremia, and need of early adjustment (within the first 3 days) in antimicrobial treatment. *Methods.* In an observational prospective exploratory study, we evaluated the

behavior of these inflammatory markers in 37 episodes of febrile neutropenia occurring in 27 hospitalized onco-hematologic adult patients (median age: 39, 78% were man). Blood samples were obtained in three different moments of each neutropenic episode: at the first day of fever (Day 0), at Day 1 and Day 3 following the first record of fever. *Results.* Procalcitonin measured at Day 0 (cutoff value 2.27 mg / L; P=0.03), sTNFRII measured on Day 1 (cutoff value: 3,740 pg/mL; P=0.05), the delta Day 3-Day 1 of IL-8 (cutoff +320 pg/mL; P=0.05) and the delta Day 3-Day 1 of eotaxin (cutoff: 43 pg/mL; P=0.03) were significantly higher in patients who died at Day 28 of follow-up as compared to their surviving counterparts. Early adjustment on antimicrobial treatment was associated with a higher Day 3 level of IL-8 (cutoff value 217.9 pg/mL; P=0.05), sTNFRII (cutoff 3,992 pg/mL; P=0.04) and MCP-1 (cutoff 1,522 pg/mL; P=0.008), all of them measured at Day 3. No inflammatory marker was associated with bacterial blood stream infection. *Conclusions.* In this exploratory study, PCT, IL-8, sTNFRII, MCP-1 and eotaxin seem to be useful markers to assess the risk of death and the need of early adjustment on antimicrobial treatment in febrile neutropenia onco-hematologic patients. These results should be confirmed in a larger cohort of patients.

1894

USE OF INFLAMMATORY MARKERS TO PREDICT OCCURRENCE OF FEVER IN HEMATO-ONCOLOGY PATIENTS DURING NEUTROPENIA

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Background. Febrile neutropenia remains the most frequent complication in hemato-oncology patients receiving chemotherapy. Fever, however, might be a late sign of infection, with consequent delay in onset of antibiotic therapy. *Aims.* We hypothesize that change in serum level of cytokine/inflammatory markers might precede occurrence of fever in neutropenic patients, allowing an earlier onset of antibiotic therapy. Fever was defined as a single axillary temperature of ≥38°C or axillary temperature of ≥37.8°C over one hour. *Methods.* In an observational prospective single centre study, we evaluated the behavior of serum tumor necrosis factor alpha (TNF-α), soluble receptors of TNF-α (sTNFRI and sTNFRII), CCL2 (MCP-1), CCL3 (MIP-1α), CCL11 (eotaxina), CXCL8 (IL-8), CXCL10 (IP-10) and procalcitonin in 32 episodes of neutropenia occurring in hemato-oncology patients. These markers were tested in blood samples obtained in the first day of neutropenia, in the day before fever and in the day of fever were tested for these markers. Median age was 36 (18-69) yrs, and acute myeloid leukemia was the most frequent diagnosis (9 patients). Ten episodes occurred in patients who underwent hematopoietic stem cell transplantation. Eight out of the 32 episodes of neutropenia remained afebrile during the follow-up (28 days) and were considered our control group. Informed consent was obtained for all patients included in this study. *Results.* sTNFRI levels, measured a median of 11 hours (1-15 hours) before the first episode of fever, were significantly higher in febrile patients as compared to patients of control group (P=0.02). Similar results were observed for sTNFRI and CCL2 levels (P=0.04 for both markers) in the subgroup analysis of non-transplanted patients. A cut-off of 1514 pg/mL for sTNFRI was able to discriminate between neutropenic patients that presented or not fever, with sensibility of 65%, specificity of 87%, PPV of 93% and NPV of 46%. *Conclusions.* Daily measurement of sTNFRI could predict occurrence of fever in onco-hematological neutropenic patients, with low sensitivity and high specificity. CCL2 might also be useful in non-transplanted patients. A large prospective trial is needed to confirm these data.

1895

PULMONARY ASPERGILLOSIS IN ADULTS WITH ACUTE LEUKEMIA (AL) DURING INDUCTION TREATMENT: REPORT OF 32 CASES AMONG 114 AL PATIENTS

M Bellaaj

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Introduction. Pulmonary aspergillosis is a frequent cause of mortality in patients affected by acute leukaemia and receiving intensive therapies. The aims of our study is to present the incidence and diagnostic difficulties of pulmonary aspergillosis during induction treatment in 114 adults patients with acute leukaemia, and to evaluate management and treatment outcomes of this fungal infection in our department. Patients and methods.This is a retrospective study concerning 114 patients, 16 to 60 year old affected by acute leukaemia and admitted in the haematology department of sfax (Tunisia) for an induction course during a 5 year period (2005 - 2009). For each patient, a file has been filed, and the following data are inscribed: disease characteristics, therapeutic modalities, infections complications based on radiologic (thoracic radiography and/ or tomodensitometry) and microbiologic (aspergillary antigenemia by ELISA test+/- sputum and broncho-alveolar washing liquid when possible) examinations. Aspergillosis diagnosis is established according EORTC/MSG group criteria, with three levels of certainly: proved, probable and possible. Voriconazole was used in probable and proved aspergilosis treatment and ampho B in possible aspergilosis treatment. *Results.*We collected 39 ALL and 75 AML cases. 32 Patients (28 % of cases) presented pulmonary aspergillosis. The diagnosis was probable in 13 patients (40%), possible in 17 patients (53%) and proved (by histologic examination) in only 2 patients (6%). All patients had a suggesting clinical picture, with prolonged fever under wide spectrum antibiotics with sometimes respiratory symptoms. Radiologic examination, aspergillary antigenemia and mycological examination were positive in 90%, 46%, and 12% of cases respectively. The evolution was favorable among 42% of patients treated by Ampho B and 69% of patients treated by voriconazole. Mortality due to aspergilosis in our patients is of 38%. It was constant in our tree patients who presented this infection during rescue curse. *Conclusion.* Aspergillosis in a acute leukaemia patients under invasive chemotherapy sets diagnostic and therapeutic problems in our study as well as in the literature. Insufficiency of our explorations (precocious tomodensitometry, broncho-alveolar washing) explains uncertainly of diagnosis (half of our cases are possible), and incites us to improve our check-up procedures, which would allow more precious diagnosis and treatment.

1896

SERUM CORTISOL IN HEMATOLOGICAL PATIENTS WITH NEUTROPENIC FEVER: IS THERE ADRENAL INSUFFICIENCY?

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Background. There are no data on the level of cortisol with respect to the inflammatory response in hematological patients with neutropenic fever. The aim of this study was to evaluate the association of serum cortisol with the level of C-reactive protein (CRP) and the development of severe sepsis in hematological patients with neutropenic fever. *Aims.* Altogether 69 hematological patients with 93 periods of neutropenic fever were included in this prospective study. Nineteen patients received therapy for acute myeloid leukemia and 50 patients were autologous stem cell transplantation recipients. *Methods.* Each period of neutropenic fever was classified as severe sepsis or not severe sepsis. Serum cortisol and CRP were determined at the onset of fever on day 0 and at 7-8 a.m. on days 1 to 4. *Results.* Serum cortisol level correlated positively with serum maximal CRP level during days 0 through 4 in neutropenic fever periods without severe sepsis, but no correlation was observed in fever periods with severe sepsis. Belonging to the lowest quartile range of increase of cortisol from day 0 to 1 was associated with high maximal CRP. *Summary/Conclusions.* In hematological patients with neutropenic fever but without severe sepsis the level of cortisol was associated with the severity of infection measured as maximal CRP. However, in fever periods complicated by severe sepsis the cortisol response was clearly attenuated. This discordance may reflect adrenal insufficiency in severe sepsis.

1897

CORRELATION STUDY BETWEEN THE D-DIMERS AND THE INFECTIONS, AND INVESTIGATION OF THEIR PROGNOSTIC VALUE IN THE OUTCOME OF THESE CASES

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Background-Aim. To investigate the relation of d-dimers to the infec-

tions and to estimate their prognostic value in the final outcome of the cases with increased levels. *Material-Method.* The material of the retrospective study included 108 cases of patients (62 men and 46 women), with the average age of 56,8 years. For all of these patients, the D-dimers blood level was measured, their clinical condition was studied, all the necessary laboratory tests were done, and after the registry of their final outcome, followed the statistical process x2. *Results.* It was proved that it considered 47 cases of infection, of which, 37 presented a moderate increase of d-dimers (220-1000 μg/L), 6 cases presented very high levels (>1000 μg/L), and only 4 patients had normal levels (<220μg/L). Out of the 6 patients with d-dimers level >1000 μg/L, 2 suffered from septicemia and passed away, and 4 suffered from intra-abdominal infections, of which 3 also passed away (pancreatitis, cholangitis). On the contrary, of the rest 37 with moderate increase of d-dimers, only one patient passed away, while no death was mentioned for patients with normal levels. Moreover, 3 cases of thrombo-embolic episodes (2 of them mortal) were registered with d-dimers level >1000 μg/L. *Conclusions.* It is, therefore, proved that: a)In addition to the septicaemia, the pulmonary embolism and the cancer, a clear increase of the level of d-dimers is also found in intra-abdominal infections, without nevertheless considering this parameter as an infection's diagnostic indicator. b)There is a strong correlation between the level of d-dimers and the cases of infections (91,5%), because of the activation of the exogenous coagulation system by the endotoxins, and finally, c)The major increase of the level of d-dimers represents undoubtedly an important (83,4%) aggravating prognostic factor for the outcome of patients hospitalized because of infection.

1898

STUDY OF COAGULATION DISORDERS DURING ACUTE FEBRILE INFECTIONS

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Background-Aim. To study and document the possible disorders in the coagulation mechanism during acute febrile infections, given the fact that the inflammatory response which follows, is directly related to the activation of the coagulation mechanism. *Material - Method.* 86 patients were studied (47 male and 39 female), aged from 24 up to 78 years of age, who presented acute febrile infection, mainly of the respiratory and urinary system (24 and 17 cases correspondingly), and also of other systems (gastrointestinal, skin and soft tissue, etc). All the patients during the course of the disease (Stage A), were tested for the usual lab parameters. The coagulation mechanism was tested using automatic analyzers, with the help of tholosimetry: measurement of Prothrombin Time PT, of International Normalized Ratio INR, of activated Partial Thromboplastin Time aPTT and Fibrinogen FIB was performed. The coagulation mechanism was retested, in patients who even had one of the above mentioned parameters influenced, after the course of the disease (Stage B). The results were compared and statistically processed. *Results.* During Stage A, only 10 patients (11.6%) had no affected parameter of the coagulation mechanism. 19 patients presented INR>1.2 (22.1%), 18 presented aPTT >37 (20.9%), while FIB>400 mg was documented in 57 cases (66.3%). The corresponding measurements during Stage B showed that: 3 patients presented INR>1.2 (3.5%), 2 patients presented aPTT >37 (2.3%), while FIB>400 was documented in 8 cases (9.3%). *Conclusions.* It is proven, that in patients with acute febrile infection, the underlying inflammatory response, causes an activation of the coagulation mechanism, which results in the overwhelming majority of patients (88.4%), to present at least a parameter with disorder. On the contrary, after the passage of the disease, in the majority of patients all the coagulation parameters return to their previous normal levels.

1899

SCREENING OF FUNGEMIA USING CELL POPULATION DATA OF AUTOMATIC BLOOD CELL ANALYZER

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Background. Recently, fungemia(fungal sepsis) has increased dramatically due to marked increase in the pool of patients at risk. Candida species are the most common cause of fungal infection affecting immunocompromised patients and are the 3rd to 4th most common pathogen recovered from blood culture. It is associated with high morbidity and mortality and the prompt diagnosis and appropriate initiation of antifungal therapy are very important. The only available method to make diagnosis of fungemia is blood culture which needs at least 1 day of time. Fungemia often cause morphologic changes in the circulating white blood cells (WBC). Recently, Coulter DxH800 show many cell polulation data (CPD) of each WBC subpopulation. *Aims.* To find out the differences of CPD between fungemia and normal subjects and determine the usefulness of CPD as a screening marker of fungemia *Methods.* One hundred thirty four subjects without hematological disorders, 65 males and 69 females aged from 3 to 80 years old, were enrolled as normal control. Twenty seven patients with fungemia, 13 males and 14 females aged from 0 to 94 years old, were selected when routine blood culture showed growth of yeasts and their WBC count is over 1,000/μL. The isolated organisms were Candida species in 18 patients (C. albicans in 4 patients) and yeasts, not identified, in 9 patients. Routine blood culture was performed using one aerobic and one anaerobic culture bottles and automatic blood culture system (Bactec Fx, BD). Identification of the grown organisms were performed using automatic microorganism identification system (Vitek II, Biomeieux). If yeasts were not successfully identified using this system, identification was done using API 20C (Biomeieux). The EDTA-anticoagulated blood samples were analyzed using Coulter DxH800, within 8 hours after collection using DxH800, collected the CPD data of WBC subpopulation, and compared with that of normal control and the previously reported data of 117 cases of bacteremia. The study was approved by the Catholic Medical Center Institutional Review Board. *Results.* There is significant difference between fungemia and normal control in MN-V-NE, SD-V-NE, SD-C-NE, MN-MALS-NE, SD-MALS-NE, MN-LMALS-NE, SD-LMALS-NE, MN-LALS-NE, SD-LALS-NE, MN-AL2-NE, SD-AL2-NE, MN-V-LY, SD-V-LY, SD-C-LY, SD-MALS-LY, MN-UMALS-LY, SD-UMALS-LY, SD-LMALS-LY, MN-LALS-LY, SD-LALS-LY, MN-AL2-LY, SD-AL2-LY, MN-V-MO, SD-V-MO, SD-C-MO, SD-MALS-MO, SD-UMALS-MO, SD-LMALS-MO, MN-LALS-MO, SD-LALS-MO, SD-AL2-MO, MN-MALS-EO, MN-UMALS-EO, MN-LMALS-EO, MN-LALS-EO, MN-AL2-EO ($P<0.05$). Receiver operating characteristic (ROC) curves showed best sensitivity and specificity in SD-V-MO(at 23.32 sensitivity 96.3% specificity 95.5%) and lymphocyte SD parameters; sensitivity from 94 to 97%, and specificity from 92 to 96%. The MN-V-MO showed good sensitivity (at 182.5, sensitivity 88.9% specificity 97.8%). The neutrophil CPD showed sensitivity and specificity from 81% to 89%. There is significant difference between fungemia and bacteremia in MN-V-NE, SD-C-LY, SD-MALS-LY, SD-UMALS-LY, SD-LMALS-LY, SD-LALS-LY and SD-UMALS-MO ($P<0.05$). ROC curves showed best sensitivity (74.1%) and specificity (72.4%) at 12.6 of SD-LALS-LY. *Conclusion.* The CPD of neutrophils, lymphocytes and monocytes could be a very useful parameter of fungemia and could be differentiated from bacteremia also. Prediction of fungemia may be much more improved by incorporating the various significant CPD into an algorithm. It could help early detection and treatment of fungemia in patients.

1900

FEBRILE NEUTOPENIA EPISODES IN PATIENTS WITH MULTIPLE MYELOMA AFTER AUTOLOGOUS HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Backgroud. Febrile neutropenia (FN) is a common and potentially life-threatening complication in patients with hematological malignancies. In this study, we analyse the characteristics of infectious complications occuring early after autologous hematopoietic stem cell transplantation (AHSCT). *Methods.* This is a retrospective study of FN episodes in multiple myeloma (MM) patients, who underwent AHSCT between Septembre 2003 and October 2009. Anti-infectious prophylaxis consisted of intensive hygiene measures without antibacterial chemoprophylaxis. Patients with FN received empirical antibiotic therapy with piperacilline-tazobactam and ciprofloxacine. Second-line antibiotic therapy consisted of a glycopeptide or amphotericin B. *Results.* A total of 93% of patients (93/100) developped FN after ASCT at median day +7 (range, 0-17). The median age of patients was 52 years (range, 32- 63). The median total duration of severe neutropenia was 7 days (range, 3-18). There were 67 (72%) FN of unknown origin, 10 (10.7%) FN clinically documented (5 pneumonias) and 16 (17.3%) FN microbiologically documented (8 bacteremias, 3 catheter-related blood steam infections, 2 skin infections, 1 urinary tract infection and 2 probable invasive pulmonary aspergillosis). The organisms isolated were 7 gram-negative bacteria (Pseudomonas aeroginosa (n=3), Acinetobacter baumanii (n=1),

Sphingomonas maltophilia (n=1), Proteus mirabilis (n=1) and Cryseomonas luteola (n=1)) ; 6 gram-positive bacteria (4 coagulase-negative staphylococcus, 1 Staphylococcus aureus and 1 Corynebacterium) and 1 Candida tropicalis (fungemia). Response to first-line empirical antibiotic therapy was seen in 77 (82.7%) FN patients. Median time to defervescence was 3 days (range, 1-5). Overall, 53 (56.9%) of the 93 patients needed at least a second-line antibiotic regimen based on glycopeptide and anti-fungal agents in respectively 28 (52.8%) and 12 (22.6%) cases. Response to second-line was obtained in 40 (74.4%) FN episodes. Infection- related mortality was 1% (1 case). *Conclusion.* ASCT should be considered a low-risk procedure in MM despite the slightly higher incidence of gram-negative and fungal infections observed in our study.

1901

POSITIONAL PARAMETERS IN HUMAN IMMUNODEFICIENCY VIRUS INFECTION (HIV)

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Background. The Coulter LH 780 hematology analyzer (Beckman Coulter, Fullerton, CA) has the ability to measure specific parameters of lymphocyte and monocyte populations like mean and standard deviation (SD) of cell volume (MVI,SDVI), conductivity (MCI,SDCI), and light scatter (MSI, SDSI). These so-called positional parameters (PP) can detect changes in lymphocyte and monocyte populations in HIV patients. *Aims.* Changes in PP of lymphocytes and monocytes in HIV patients compared to control group. *Methods.* Lymphocytes and monocytes PP data from 21 HIV patients and from 53 age-matched healthy control subjects were prospectively analyzed. The PP was obtained by the Coulter LH 780 hematology analyzer. Comparisons between means were performed by analysis of variance. Comparison between 2 means was performed by using the Student t test. A P value less than 0, 05 were considered significant. *Results.* Table. *Conclusions.* 1)HIV infection is characterized by increased MVI and decreased MSI in both lymphocyte and monocyte populations. These results are statistically significant. 2) SDVI and SDSI also significantly increased both in lymphocytes and in monocytes compared with controls despite the fact that patients are on anti-retroviral therapy. 3)For the potential clinical utility of these findings in monitoring HIV patients we need prospective clinical trials that include untreated patients.

Table.

Mean	Control lymph.	Patient lymph.	p	Control monoc.	Patient monoc.	p
MVI	83.8	87.5	0.004	167.6	175.5	0.0001
MCI	115	114.9	0.8	122.2	122.1	0.76
MSI	75.2	68.1	0.0001	93.5	86.6	0.0001
SDVI	14.9	15.9	0.019	17.6	20.7	0.0001
SDCI	13.4	12.2	0.034	4.8	4.8	0.8
SDSI	17.8	18.6	0.031	9.5	10.8	0.0001

1902

DETERMINATION OF THE SEROLOGICAL PROFILE OF CHILDREN IN GREECE WITH INFECTIOUS MONONUCLEOSIS IN THE NORTH GREECE

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Background-Aim. To determine the serological profile of children with suspicion of Infectious Mononucleosis (IM) and to document the frequency of this infection (which in many cases is asymptomatic), as well as the epidemiological characteristics of IM. *Material-Methods.* A total of 176 children were studied, (92 boys and 84 girls), up to 17 years of age, with symptoms suspicious for Epstein-Barr virus infection. The ELISA method was used to look for specific antibodies against the capsid of the virus VCAIgG and against the nuclear antigen EBV-IgM, while taking into consideration the possible increase of the VCAIgG title between two serum samples. *Results.* Totally, 51 positive cases of children were found (29%) with active infection : 28 boys (14 <5 years of age, 12 5-10 years of age and 2 >10 years of age) and 23 girls (10 <5 years of age, 6 5-10 years of age and 7 >10 years of age). Pharyngitis was present in 47 children (92,2%), 39 had fever (76,5%) and 48 had lymphadenitis (94%). The lab tests revealed leukocytosis up to 20.000 leukocytes in 29 cases (56,9%) and leukocytosis >20.000 in 9 cases (17,6%). The most frequent complication documented was streptococcal superinfection in 13 children (25,5%) and thrombocytopenia in 8 children (15,7%). A past infection (negative EBV-IgM values and positive VCAIgG values) was documented in 24 children and teenagers (14%), while 101 children (57%), mainly of little age, were negative for infection. *Conclusions.* Therefore, it is proven that : 1)Epstein-Barr virus infection is common among children and teenagers. 2)Serum negative are mainly the children of little age and 3)There is no statistically important difference between the two sexes, while on the contrary there is a seasonal distribution of the infection, with winter and summer outbreaks.

1903

C-REACTIVE PROTEIN IS A USEFUL TOOL FOR PREDICTION OF NEUTROPENIC SEPSIS WITH OR WITHOUT FEVER

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Background. Neutropenic infection is a serious complication of chemotherapy requiring prompt intervention. A test permitting early diagnosis could reduce diagnostic uncertainty or permit studies of early intervention. C-reactive protein (CRP) is a widely available, sensitive marker of inflammation and infection. *Aims.* To determine if CRP can predict the development and course of neutropenic infection. *Methods.* Episodes of post-chemotherapy neutropenia (neutrophil count $<0.5\times10^9$/L) lasting at least 5 days between December 2008- January 2010 were retrospectively identified from a laboratory database. Patient records were reviewed for fever, corticosteroid use, presence of disorders causing CRP elevation, antimicrobial administration, blood cultures and daily CRP *Results.* Data was collected for the following categories - Febrile Neutropenia (FN): fever >38°C but not already receiving systemic antimicrobials. Afebrile Infected Neutropenia (AIN): no fever but given systemic antimicrobials for clinically detectable infection. Uninfected Neutropenia (UN): no fever or systemic antimicrobials. Day 0 (D0) was the day of antimicrobial initiation (FN, AIN) or of maximum CRP (UN). *Results.* 50 FN, 8 AIN and 30 UN episodes were identified, 6 further episodes with incomplete CRP data were excluded. For all AIN and 48/50 FN episodes there was daily CRP rise from D-3 to D-1 with maximum CRP on D-1. UN episodes had no consistent pattern. Only 3 UN episodes had CRP>40 mg/L, one with extensive drug rash (CRP 41), one with tissue damage (60), and one otherwise well (48). One AIN and 6 FN episodes had maximum CRP<50. In two of these the patient was receiving oral corticosteroids (CRP 41 and 42). CRP>50 was therefore sensitive and specific for systemic antimicrobial requirement. However, the time from CRP >50 to antimicrobial requirement varied from 1 to 11 days. This variation was explained by inclusion of 10 FN episodes in patients with concomitant non-infective causes of CRP elevation, most commonly tissue trauma and severe drug reaction, who became febrile 7-11 days after CRP>50. In this group a CRP>50 alone, given the time to fever, probably does not equate to infection. However, a >50% CRP rise over two consecutive days was seen in all these episodes between D-1 to D-4. A 50% change alone does not discriminate well between UN and uncomplicated FN or AIN because of the small absolute value changes in some patients. Combining a minimum CRP>50 with a rise of >50% over 2 consecutive days produces a sensitivity of 86% and specificity of 100% for the subsequent development of neutropenic fever / infection within 3 days in all patients. After antimicrobial initiation, failure of CRP to fall >20% between D0 and D+3 correlated with, but did not predict, persisting fever at 48 hours and second line antimicrobial use. No CRP parameter correlated with positive blood cultures. *Conclusion.* A minimum CRP threshold of 50 mg/L combined with a minimum rate of rise of 50%

over 2 consecutive days is a highly sensitive and specific predictor of the need for systemic antimicrobials within 3 days. CRP values are not useful for predicting response to first line antimicrobials or organism isolation.

Table. Summary of CRP data.

	UN	FN	AIN	FN+AIN	FN+AIN simple	FN+AIN complex
Number of episodes	30	50	8	58	48	10
CRP on D-1 (D0 for UN) Median (range)	11 (<3-60)	74 (10-375)	67 (41-114)	80 (10-375)	91 (10-265)	308 (114-375)
CRP>50 No. (%)	1(3)	44(88)	6(75)	50(86)	46(97)	10 (100)
Median Day CRP >50	-4	-4	-1	-2	-2	-9
Predictive value of CRP>50	Sensitivity 86% / Specificity 97% / time to fever/antibiotics 1-11 days					
CRP rise of >50% over 2 days No. (%)	11 (27)	48 (96)	8 (100)	56 (97)	46 (96)	10 (100)
Days to antibiotics Median (range)	NA	2 (1-3)	2 (1-3)	2 (1-3)	2 (1-3)	2 (1-3)
Predictive value of CRP rise >50%	Sensitivity 97% / Specificity 63% / time to fever/antibiotics 1-3 days					
Min CRP>50 & CRP rise >50% No.(%)	0 (0)	44 (88)	8 (100)	50 (86)	46 (96)	10 (100)
Predictive value CRP >50 & CRP rise>50%	Sensitivity 86% / Specificity 100% / time to fever/antibiotics 1-3 days					

1904

H1N1 ASSOCIATED HEMOPHAGOCYTIC SYNDROME SHOULD BE TAKEN INTO ACCOUNT

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We are presenting the case of a 14 year-old girl without any underlying disease who developed H1N1 associated hemophagocytic syndrome. Persistant fever without any evidence of secondary bacterial infection under antiviral treatment, hyperferritinemia and organomegaly are signs of suspected hemophagocytic lymphohistiocytosis (HLH) in H1N1 patients. To our knowledge, this is the first documented case of H1N1 complicated hemophagocytosis in a previously healthy child, and clinicians should be aware of the possibility of HLH. *Case.* A fourteen-year-old girl presented to our emergency room with complaints of fever, cough and progressive breathing difficulty for three days. She had no previously known chronic illness and her past history was unremarkable for infections and vaccinations. She was admitted to our infectious disease department with suspected H1N1 infection due to her contact with H1N1 (+) people. Upon physical examination, she was dyspneic, tachypneic and confused. Her fever was 39°C. The respiratory rate was 46/minute. Arterial pressure was 110/80 mmHg. Inspiratory crackles and wheezing were heard on auscultation. Her saturation was 56% without oxygen support. She had no organomegaly and lymphadenopathy. The initial hemogram showed: Hb: 12.8 gr/dl, WBC: $1{,}8\times10^9$/L with the 40% polymorphonucleated cells, 20% band, 20% lymphocyte and 12% monocyte, and a low platelet count (89×10^9/L). Her chest X-ray revealed diffuse, bilateral infiltration. Broad-spectrum antibiotherapy was immediately started. Because of the outbreak, a nasopharyngeal swab for H1N1 was also taken and oseltamivir was added to the treatment.She was intubated due to progressive respiratory distress, hypoxic encephalopathy and the presence of progressive respiratory acidosis on blood gases analysis at the 24th hour of admission.On the third day, H1N1 was positively diagnosed by polymerase chain reaction. During a clinical follow-up, hepatomegaly was detected on the thirteenth day. Several sets of blood, urine and stool cultures were all negative for pathogenic microorganisms. Although complete blood counts showed no cytopenia, the development of hepatomegaly and a persistant fever with spikes to 39°C made us suspect hemophagocytosis. Bone marrow aspiration was performed with a formulation of the bone marrow smears as follows: 40% normoblast, 3% promyelocyte, 16% myelocyte, 12% metamyelocyte, 5% band, 17% polymorphonuclear leukocytes, 4% lymphocyte, 3% monocyte. A bone marrow investigation also disclosed activated histiocytes with engulfment of normoblasts, erythrocytes and platelets. Serum ferritin and triglyceride levels were 1446 ng/mL and 215 mg/dL, respectively. Fibrinojen level was 513 mg/dL. Decreased NK level (CD16+56: 2%) was shown by flow-cytometer. No improvement occured in her clinical condition and hemophagocytic macrophages were shown in the aspirate from the endotracheal tube, as well as IVIG therapy. Therefore, high dose methylprednisolone (10 mg/kg/d) was started. Her body temperature normalized and serum ferritin levels decreased 2 days after steroid treatment. Cyclosporine and etoposide were added to her treatment according to HLH-2004 protocol. Infiltrations on chest X-ray apparently regressed and the pressure limits of mechanical ventilator were decreased. Our patient is still on mechanical ventilation and her treatment is ongoing. Because of the consanguineous marriage of her parents (1st cousins), hemaphagocytic lymphohistiocytosis mutation analysis is planned.

1905

ABSOLUTE NEUTROPHIL VOLUME DISTRIBUTION WIDTH CORRELATION TO NEUTROPHIL COUNT IN ACUTE INFECTION

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Background. Review of peripheral blood smears can yield important diagnostic information through the identification of the morphologic changes characteristically seen in reactive neutrophils during infection. This approach, however, is labor-intensive and time-consuming because it requires manual examination. Furthermore, the results are subjective because they depend on human interpretation, and only a few hundred cells can be analyzed for any given sample. The Coulter LH 780 hematology analyzer (Beckman Coulter, Fullerton, CA) has the ability to measure specific parameters of neutrophil populations like mean and standard deviation (SD) of cell volume (MVI,SDVI), conductivity (MCI,SDCI), and light scatter (MSI, SDSI). These so-called positional parameters (PP) can detect morphologic changes in neutrophil population and can be an additional indicator for diagnosing acute infection. *Aims.* To investigate the value of the neutrophil SDVI generated by VCS technology of the Coulter LH 780 hematology analyzer, as an additional predictor of acute infection. *Methods.* Absolute neutrophils count, and SDVI data from 552 patients with positive blood cultures and from 54 age-matched healthy control subjects were prospectively analyzed. We then studied whether changes in SDVI correlated with patients absolute neutrophil counts (less or greater than 6600/μL). The PP was obtained by the Coulter LH 750 hematology analyzer. Comparisons between means were performed by analysis of variance. Comparison between 2 means was performed by using the Student t test. A P value less than 0, 05 were considered significant. *Results.* A significant increase in the SDVI was observed in the bacteremic patients compared with the controls (28, 1 *vs.* 20, 6, P<0,001). Such increase was observed even in patients with absolute neutrophil counts less than 6600/μL) (25, 7 *vs.* 20, 6, P<0,001). The more dramatic increases were seen in patients with neutrophilia (29, 1 *vs.* 20, 6, P<0,001). Table. *Summary/Conclusion.* 1) The SDVI increases in acute infection. 2) Using an SDVI cutoff of 23, as in bibliography, seems that SDVI is a good predictor of acute infection. 3) The SDVI increase correlated significantly with neutrophilia but was observed even in patients with neutrophil counts less than 6600/μL. 4) As a quantitive parameter, the SDVI has potential use as an additional indicator for diagnosing acute infection.

Table.

	Control	Patients	<6600	>6600	P
Number	54	552	156	396	
SDVI mean	20.6	28,1	25.7	29,1	<0,003

1906

POSITIONAL PARAMETERS: INDICATORS OF ACUTE INFECTION

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Background. Review of peripheral blood smears can yield important diagnostic information through the identification of the morphologic changes characteristically seen in reactive neutrophils during infection. This approach, however, is labor-intensive and time-consuming because it requires manual examination. Furthermore, the results are subjective because they depend on human interpretation, and only a few hundred cells can be analyzed for any given sample. The Coulter LH 780 hematology analyzer (Beckman Coulter, Fullerton, CA) has the ability to measure specific parameters of neutrophil populations like mean and standard deviation (SD) of cell volume (MVI,SDVI), conductivity (MCI,SDCI), and light scatter (MSI, SDSI). These so-called positional parameters (PP) can detect morphologic changes in neutrophil population and can be an additional indicator for diagnosing acute infection. *Aims.* To investigate the values of neutrophils PP generated by VCS technology of the Coulter LH 780, as additional predictors of acute infection. *Methods.* Neutrophils PP data from 552 patients with positive blood cultures and from 54 age-matched healthy control subjects were prospectively analyzed. The PP was obtained by the Coulter LH 780 hematology analyzer. Comparisons between means were performed by analysis of variance. Comparison between 2 means was performed by using the Student t test. A P value less than 0, 05 were considered significant. *Results.* 63,9% patients were male and 36,1% females. 83% positive blood cultures had 1 microorganism, 14,4% 2 and 2,6% had 3. Gram (+) microorganisms were found in 34, 8% and gram (-) microorganisms in 59,5% positive blood cultures. When we studied full blood count we found that 28, 3% had less than 6600/μL neutrophils and 71,7% more than 6600/μL neutrophils. SDVI between 1 or 3 microorganisms correlated significantly (P=0,045) but the number of 3 microorganisms in positive blood cultures was small (452 v 14). Table. *Conclusions.* 1) MVI and SDVI increase in acute infection. 2) MCI and MSI decrease in acute infection. 3) Using an SDVI cutoff of 23 and MVI cutoff of 150, as in bibliography, seems that SDVI and MVI are good predictors of acute infection. 4) As quantitive parameters, PP have potential use as additional indicators for diagnosing acute infection.

Table.

	Control	Patients	P
Number	54	552	
MVI mean	146.17	162.71	<0,0001
MCI »	145.85	140.89	<0.0001
MSI »	146.06	136.57	<0.0001
SDVI »	20.63	28.10	<0.0001
SDCI »	6.43	8.05	<0.0001
SDSI »	11.79	12.35	0.048

1907

TOXOPLASMOSIS AS A CAUSE OF PANCYTOPENIA IN IMMUNOSUPPRESSED PATIENTS

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Introduction. Latent infection by Toxoplasma gondii affects millions of persons worldwide, but rarely causes significant disease in immunocompetent patients. However, in recipients of solid organ transplants it can cause significant morbidity and mortality. Reactivation of latent infection can occur during use of immunosuppressive medications, and majority of patients are asymptomatic. Development of symptomatic infection by Toxoplasma gondii is associated with a high mortality (~65% in recent series). A few reports suggest that Toxoplasma gondii can cause pancytopenia in immunocompromised patients. We describe two patients post-kidney-pancreas transplant who developed symptomatic toxoplasmosis involving the bone marrow. *Objective.* To describe two immunocompromised patients who developed pancytopenia secondary to Toxoplasma gondii infection. One of the cases was previously reported (Seguro FS *et al.,* Brit J Haematol, 2009, 147:276). *Methods and results.* Both patients were receiving tacrolimus, mycophenolate mofetil and methylprednisolone for immunosuppression after kidney-pancreas transplant. Case#1 was a 33 year-old male who developed fever, headache and tachypnea 3 months after transplantation. The patient was receiving treatment with ganciclovir for cytomegalovirus reactivation at the time. He developed pancytopenia and bone marrow biopsy revealed a hypocellular marrow with increased macrophagic activity. Afterwards, he developed microangiopathic anemia that didn´t improve with plasma exchanges and discontinuation of tacrolimus. His bone marrow was reevaluated and trophozoites suggestive of Toxoplasma gondii were identified and confirmed by immunohistochemistry. A CT scan and MRI of the brain didn´t show a mass lesion, and cerebrospinal fluid analysis was negative for Toxoplasma gondii by polymerase chain reaction. Toxoplasma gondii serology was positive for anti-toxoplasma IgM. The patient passed away before the diagnosis could be confirmed, so no specific treatment was administered. Case#2 was a 40 year-old male who was admitted 4 months post-transplant for fever of unknown origin and anemia. He had no neurologic symptoms. After 5 days the patient became pancytopenic, and a bone marrow evaluation showed Toxoplasma gondii trophozoites which were confirmed by immunohistochemistry. He received treatment with sulfadiazine-pyrimethamine but died 2 months later. *Conclusion.* Toxoplasmosis occurs due to reactivation of latent foci or from primary infection, and can cause a myriad of symptoms in immunocompromised patients, including pancytopenia. The parasite can be identified in bone marrow smears and bone marrow biopsies. Bone marrow evaluation with specific staining for Toxoplasma gondii trophozoites is indicated in immunocompromised patients post-solid organ transplant who develop pancytopenia, particularly in populations where the prevalence of infection is high.

1908

USE OF LIPID AMPHOTERICIN B (ABELCET) IN HAEMATOLOGICAL PATIENTS SUBMITTED TO ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANT: A SINGLE CENTRE EXPERIENCE

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Background. In haematological patients a persistent high-grade fever that does not improve when treated with antibacterials is often assumed to be due to fungal infection. The approaches to the treatment of fungal infections include several lipid formulations of Amphotericin B such as Ambisome and Abelcet and other molecules including Caspofungin and Voriconazole. *Aims.* To evaluate a single centre experience with Abelcet use in a group of haematological patients who had undergone allogeneic hematolopietic stem cell transplant (HSCT). *Methods.* This retrospective study evaluated 11 patients (7 males, 4 females), 17-54 years old (median age 31) with neoplastic haematological diseases who had recently undergone HSCT at a median time from diagnosis of 9 months. The conditioning regimen was myeloablative in 10 cases and an anti-lymphocyte treatment was associated in 3 cases. In the post-HSCT phase, during the neutropenic period (duration13-30 days, median 17), fever (BT ≥38°C) appeared in 10/11 cases after a median of 5 days (range 1-22) from HSCT. On the basis of microbiological studies and imaging (X-ray, CT, and ultrasound) the fever was associated with pneumonia in 2 cases and sepsis in 5, while it remained of undetermined origin in 3 cases. A poli-antibiotic therapy was administered in the 10 febrile patients. *Results.* Antifungal treatment with Abelcet was started at a median distance of 7 days (range 3-24) from HSCT in all 11 patients, as secondary prophylaxis in the apyretic patient (180 mg/day) and, due to the persistence of the fever in the other 10 patients, as emptive or pre-emptive treatment (300 mg/day). This Abelcet treatment had a median duration of 7 days (range 4-13 days) and, after a median time of 4 days, a creatinine increase was observed in 7/11 cases with a median duration of 6 days and with a maximum level ranging from 1.42 to 2.69 mg/dL (median 1.66). In two of these 7 cases the antifungal treatment was continued with Caspofungin. No other significant adverse events related to the Abelcet treatment were

reported. At the end of the Abelcet treatment, only 3/10 patients remained febrile and the pneumonia disappeared in one of the two cases. An acute graft *vs.* host disease grade II-III was observed in six cases and the HSCT-related mortality (within 100 days) was 27% (3/11 cases; 2/3 receiving Abelcet and then Caspofungin). *Conclusions.* Antifungal treatment with Abelcet in eleven haematological patients who had undergone allo-HSCT in a single centre, performed as secondary prophylaxis in one case and as emptive or pre-emptive treatment in the other ten cases with a median duration of seven days and with a median dose of 300 mg/day, was satisfactorily tolerated and associated with a considerable reduction of infectious complications and with an acceptable HSCT-related mortality rate.

1909

POSITIONAL PARAMETERS IN DIFFERENTIAL DIAGNOSIS OF H1N1 IN CHILDREN

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Background. The H1N1 pandemic was a great public health problem in the last months of 2009 both for the world community and for Greece. The Coulter LH 780 hematology analyzer (Beckman Coulter, Fullerton, CA) has the ability to measure specific parameters of lymphocyte and monocyte populations like mean and standard deviation (SD) of cell volume (MVI,SDVI), conductivity (MCI,SDCI), and light scatter (MSI, SDSI). These so-called positional parameters (PP) can detect changes in lymphocyte and monocyte populations. *Aims.* The purpose of this study was to evaluate the utility of PP in differential diagnosis of H1N1 infection in children population. *Methods.* We studied 64 patients (<14 years old)) who came at emergency department with flu symptoms. Confirmation of H1N1 was by PCR. Full blood count and PP was obtained by the Coulter LH 780 hematology analyzer.Comparisons between means was performed by analysis of variance. Comparison between 2 means was performed by using the Student t test. A P value less than 0, 05 were considered significant. *Results.* Table. *Conclusions.* 1) The absolute number of lymphocytes in H1N1 positive patients is reduced significantly (P<0.0001). 2) The number of platelets in patients is reduced (P=0.073). 3) MVI (P=0.078) and MCI(P=0.023) are reduced in patients. Prospective studies with larger numbers of patients are needed to asses the possible clinical utility of PPs in H1N1.

Table.

Table	H1N1 (+)	H1N1 (-)	P
samples	34	30	
lymphocytes x $10^3/\mu l$	2.24	4.4	0.0001
MVI lymphocytes	79.76	82.43	0.078
MCI "	113.5	115.5	0.023
PLT	273.5	325.8	0.073
monocytes x $10^3/\mu l$	0.8	1.5	0.0001

1910

POSITIONAL PARAMETERS IN DIFFERENTIAL DIAGNOSIS OF H1N1

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Background. The H1N1 pandemic was a great public health problem in the last months of 2009 both for the world community and for Greece. The Coulter LH 780 hematology analyzer (Beckman Coulter, Fullerton, CA) has the ability to measure specific parameters of lymphocyte and monocyte populations like mean and standard deviation (SD) of cell volume (MVI, SDVI), conductivity (MCI, SDCI), and light scatter (MSI, SDSI). These so-called positional parameters (PP) can detect changes in lymphocyte and monocyte populations. *Aims.* The purpose of this study was to evaluate the utility of PP in differential diagnosis of H1N1 infection. *Methods.* We studied 277 patients, aged 8 months - 83 years, who came at emergency department with flu symptoms. Confirmation of the disease was by PCR. Full blood count and PP were obtained by the Coulter LH 780 hematology analyzer. Control group were 53 samples of healthy donors. Comparisons between means were performed by analysis of variance. Comparison between 2 means was performed by using the Student t test. A P value less than 0, 05 were considered significant. *Results.* 34 positive patients were children and 92 were adults. There were a statistical significant difference in platelet number between the two groups (children $273.5\times10^3/\mu L$ *vs.* $196.6\times10^3/\times L$ adults, P<0.0001). Table. *Conclusions.* 1) The absolute number of lymphocytes in H1N1 positive patients is reduced significantly (P<0.0001). 2) The number of platelets in patients is reduced significantly (P<0.001). The length of this reduction in adult patients (a sufficient number of those verging to mild thrombocytopenia) compared to children must be noted. 3) The absolute number of monocytes in H1N1 positive patients increases significantly (P<0.0001). 4) The absolute number of lymphocytes is reduced, but monocytes MVI and lymphocytes, monocytes SDVI are increased in patients compared to controls. Prospective studies with larger numbers of patients are needed to asses the possible clinical utility of PPs in H1N1.

Table.

	H1N1 (+)	H1N1 (-)	P	Control group	P
	136	109		53	
lymph x $10^3/\mu l$	1.48	2,36	0.0001	2.2	0.0001
MVI lymph	83,9	85,7	0.012	83.8	
SDVI lymph	16.1	16.3	0.374	14.9	0.0001
PLT	214,7	255,9	0.001		
monoc x $10^3/\mu l$	0.8			0.54	0.0001
MVI monoc	181,3			167.56	0.0001
SDVI monoc	23.6			17.58	0.0001

1911

TOXOPLASMOSIS IN CNS: OPPORTUNISTIC INFECTION WITH DIFFICULT DIAGNOSABILITY IN A PATIENT WITH IDIOPATIC THROMBOCYTOPENIC PURPURA TREATED WITH IMMUNOSUPPRESSIVES ON A LONG-TERM BASIS

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Toxoplasmosis in immunodeficient patients can have severe or even fatal progression. Laboratory diagnosis is difficult and treatment initiation must not be postponed. CNS affliction is typical, empirical treatment is used. We refer a patient case in whom the diagnosis was made on the basis of successful empiric antibiotic therapy and findings of magnetic resonance (MR). Since February 2007 a 43 years old patient with idiopatic thrombocytopenic purpura (ITP) was treated due to refractoriness of illness with substantive dose of corticoids in combination with cyklosporin A. In May 2008 a focal pulmonary diffusion process was identified on PET CT and therefore a diagnostic thoracotomy with resection of lung locus was made with histological finding of B non-Hodgkin lymphoma (B-NHL) MALT type. In August 2008 he was subfebrile and occurred cough, headaches, desorientation, visual disturbances and emesia. Neurological examination identified slight left-sided hemiparesis. The patient was meningeal when admitted into hospital. Liquor examination was negative (including PCR for Toxoplasma gondii), liquor immunophenotypization for exclusion of NHL defect was also negative. Magnetic resonance of brain identified arachnoid cyst frontally right without any signs of expansion, multiple supratentorial nidus with perifocal edema with ring enhancing imiging. Due

to toxoplasmosis suspicion a combined therapy with klindamycin 600 mg 4× per day i.v., kotrimoxazol (sulfamethoxazol and trimetoprim) i.v. in doses of sulfamethoxazol 70 mg/kg/D and pyrimetamin 50 mg/day was used. Alleviation of headaches, perplexity regression and eyesight improvement occurred within 3 days. $CD4^+$ T-lymphocytes examination from peripheral blood proved substantial lymphocyte count decrease: 0,186.109/l (referring rate: 0,44-1,8), patient is HIV 1+2 negative. Stereotactic biopsy of brain locus proved only gliosis during the therapy. After one month hospitalization the patient was released for home after-care with proven significant regression of infiltrates and perifocal edema on brain MR. Combined antitoxoplasmosis treatment was conducted for 6 weeks. Revisional lung CT in November 2008, when the patient was still asymptomatic, proved persistence of insignificant lung infiltrates and therefore transbronchial lung biopsy was indicated with histological findings of focal active inflammation of lymphocytic pneumonitis type, evaluated as postinfectious. As since June 2009 the patient observed gradual ingravescence, i.e. visual disturbances, shivering, protracted psychomotor pace, and subsequently also headaches, 1× seizure of convulsions - grand mal type, suspicion of toxoplasmosis recurrence occured. Brain CT identified three right-sided frontoparietal intraparenchym haemorrhagies with edem portion which was closed as recurrence of brain toxoplasmosis with signs of haemorrhage and therefore a combined antitoxoplasmosis therapy with pyrimetamin, klindamycin and kotrimoxazol was restarted. After 14 days significant improvement of clinical state occurred, brain MR after 4 weeks proved significant regression of findings. The invasive combined therapy continued for 8 weeks. Due to persisting immunodeficiency of $CD4^+$ T lymphocytes (0,220.10 9/L) was the risk of illness recurrence stated as high and secondary prophylaxis with pyrimetamin 25-50 mg/day in combination with klindamycin 1200 mg/day was therefore indicated. This detailed study of our patient with diagnosis of recurring CNS infection with Toxoplasma gondi was intended to demonstrate diagnostic and therapeutical issues relating to the opportunistic infection in a immunodeficient patient.

1912

BONE MARROW FAILURE , INTERSTITIAL PNEUMONIA AND COAGULOPATHY AS ONSET MANIFESTATIONS OF CHRONIC ACTIVE EPSTEIN BARR VIRUS INFECTION (CAEBV)

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CAEBV is a very rare disorder in which EBV infection results in a persistent ,ongoing viral infection of one or more organ systems. CAEBV is a disease with high mortality and morbidity with life threating complications such as virus associated hemophagocytic syndrome, interstitial pneumonia, lymphoma, coronary aneurism and central system involvement. CAEBV has been defined by Straus (Straus SE, J Infect Dis 1988) usign 3 diagnostic criteria: 1) severe illness lasting 6 months or more with markedly elevated EBV serology or chronic progressive disease that began as primary EBV infection 2) histologic evidence of major organ involvement such as interstitial pneumonia 3) increased quantities of EBV in affected tissues. Many cases have been reported not fullfilling the criteria mentioned above : Kimura reported extremely high viral loads assessed by quantitative PCR in CAEBV patients, suggesting the last one as the main diagnostic criteria (Kimura Blood 2001). We report a case of CAEBV with unusual onset. A 64 yrs old female patient was admitted on August 2009 on Internal Medicine Department because of shortness of breath lasting a few days; the full blood count showed a macrocitic anemia with thrombocytopenia: Hb= 6.7g/dL , MCV= 110fl, WBC= 9300 /μL with normal differential count, Plt= 29000/μL; chest xray showed a left pleural effusion while abdominal echography revealed liver enlargement; CT lung showed diffuse ground glass opacities, pleural effusion and consolidation with multiple air bronchograms. Ten days later, because of pulmonary function tests worsening, the patient was admitted to Intensive Care with ARDS clinical picture; after CPAP withouth any improvement the patient has been intubated. Pathogens detection (Gram+ Gram- fungi and galactomannan antigen) on sample from BAL proved negative, so were negative legionella and pneumococcal urine antigens; CMV DNA and CMV Ag pp65 were negative. A week later the patient was extubated but the following day , due to EGA and Pa O2 worsening CPAP was resumed with good recovery of respiratory tests (chest x ray showed bilateral diffuse extensive nodular shadowing); a few days later a deterioration of blood parameters was observed: Hb= 8 g/dL, WBC= 700/μL (53% granulocytes; 37 % lymphocytes) Plt = 3000/μL; bone marrow aspiration showed a severe hypoplasia ; meanwhile a low fibrinogen level (< 100 mg/dL) consistent with fibrinogenolysis was detected and the patient was managed with almost daily fresh frozen plasma infusion. Paroxismal nocturnal hemoglobinuria and thrombotic thrombocytopenic purpura were ruled out respectively with peripheral blood cytofluorimetric test (CD55 and CD59 antigens regular expression) and ADAMTS 13 level evaluation ; Evans syndrome has been excluded by Coombs test negativity , antiplatelets antibodies negativity and no platelets response to steroid and high dose immunoglobulins .EBV DNA search on bone marrow resulted positive with 1000 viral copies /mL; the same test on peripheral blood showed a strong positive result with 600 viral copies/mL; standard EBV serology performed was in keeping with previous EBV infectio: IgM and EA absent, Ab antiEBV VCA 750u/mL, antiEBNA 600 u/mL. On the basis of these results the diagnosis of CAEBV was performed; a progressive slow recovery af cytopenia and fibrinogen was observed together with clinical improvement.

1913

LONG-TERM TREATMENT OF SEVERE MYASTHENIA GRAVIS BY EXTRACORPOREAL IMMUNOGLOBULIN ELIMINATION

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Introduction. Myasthenia gravis (MG) is a neuromuscular disorder leading to fluctuating muscle weakness and fatigue. Rarely, long-term stabilization is not possible through the use of thymectomy or any known drug therapy. Plasmapheresis or some more specific hemapheretic methods can improve the status of patients even when the disease is resistant to standard therapy. *Methods and patients.* Presently, 1916 patients are treated at the Myasthenic Center in Prague. Only six of these patients were in severe condition despite thymectomy and drug therapy (including corticosteroids, immunosuppressive drugs and repeated doses of immunoglobulins) and could not be stabilized for a longer period of time. Therefore, these patients were included in the program of long-term therapy with extracorporeal elimination of immunoglobulins. We present our experience with extracorporeal immunoglobulin (Ig) elimination by immunoadsorption (adsorbers with human Ig antibodies). Acetylcholine receptor antibodies (AChRAs) were measured during long-term monitoring (4.7±2.9 years; range 1.1-8.0). *Results.* A total of 474 samples (232 pairs) were analyzed, and a drop in AChRA levels was observed (P=0.025). The clinical status of patients improved and stabilized. Roughly 6.8% of patients experienced clinically irrelevant side-effects. *Conclusion.* The method of Ig elimination by extracorporeal immunoadsorption (IA) is a clinical application of recent biotechnological advances. This hemapheretic method offers an effective and safe therapy for severe MG even when the disease is resistant to standard therapy.

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1914

HLA-G AND CORD BLOOD TRANSPLANTATION IN THALASSAEMIC SIBLINGS: MORE CHANCES FOR HIGHER TOLERANCE?

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HLA-G is a non-classical HLA class I molecule mainly expressed during pregnancy on the syncytiotrophoblast to allow maternal acceptance of the foetus. HLA-G molecule has powerful tolerogenic effects due to its immunological properties: it can inhibit the cytolysis mediated by both Natural Killer cells and antigen-specific cytotoxic T cells. In organ transplantation setting, a significant correlation has been described between heart transplant acceptance and HLA-G expression in donor's heart tissue. Not all transplanted patients expressed HLA-G, but those who produced HLA-G had a clear survival advantage and a higher protection from graft rejection. Previous studies demonstrated that a 14bp insertion/deletion polymorphism in the 3'UTR of the HLA-

G gene leads to low production of more stable subpopulation of HLA-G m-RNA. The clinical role of HLA-G 14bp polymorphism has been described in a group of adult thalassaemia patients transplanted with unrelated HLA-identical bone marrow HSC donors. Patients with the 14bp deletion/deletion genotype showed a higher risk of developing acute graft-versus-host disease (GvHD) compared to those carrying the 14bp deletion/insertion and 14bp insertion/insertion genotypes, with a relative risk increasing by three times. For this purpose, we investigated the 14bp HLA-G genotypes in 13 infant thalassaemia patients transplanted with their HLA-identical sibling Cord Blood (CB) donors (all CB units collected and cryopreserved at the Pavia CB Bank). The patient sample was composed by children comparable for age, weight, conditioning regimen, GvHD prophylaxis and CB total nucleated cell graft content infused. We performed a molecular analysis of the HLA-G genotypes of each recipient/donor couple by PCR technique. All recipients showed the same HLA-G 14bp genotype of their corresponding sibling donors. 9 patients were heterozygous (insertion/deletion), 2 were homozygous for the insertion (insertion/insertion) and 2 were homozygous for the deletion (deletion/deletion) polymorphism. None patient developed GvHD and, at present, all recipients are alive with complete neutrophils and platelets recovery within day 37 and 73 respectively. We also investigated the correlation between soluble HLA-G (sHLA-G) concentrations and HLA-G genotypes in thawed aliquots of CB plasma (stored at -190°C as the CB unit) by ELISA technique and PCR-SSP amplification respectively. No statistically significant variability was observed in CB sHLA-G levels among the 3 HLA-G genotype groups (sHLA-G average concentration 39.07 ng/mL). These results may support the hypothesis of a particular fine regulation of sHLA-G secretion during pregnancy bypassing the foetal 14pb HLA-G genotype. A quantitative sHLA-G expression screening of recipients could be a useful criterion in preventing transplant immunological adverse outcomes in unrelated adult HSC transplantation. Although preliminary for the small size of the patients sample, our findings seem to suggest that, in CB transplantation setting, the HLA-G 14bp genotype of the recipients may not influence the outcome as to rejection and GvHD, because CB may be considered an immunotolerogenic sHLA-G source per se.

1915

GRANULOCYTE TRANSFUSION THERAPY. EFFICACY TO RESOLVE THE INFECTIONS IN PATIENT WITH SEVERE NEUTROPENIA WITH IDENTIFIED GERM

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Background. A Neutropenia-related infection is the main causes of morbidity and mortality in patients with hematologic malignancy. Granulocyte transfusion therapy is used to treat neutropenic patients with severe infections which do not respond to appropriate antimicrobial agents. A renewed interest in the use of the transfusion of granulocytes in this group of patients. *Aims.* To analyze the effectives of granulocyte transfusion therapy (GTT) in patients with severe neutropenia and not controlled infections with 2 or more lines of antimicrobial agent and/or adjusted to focus or germ. *Methods.* from May of 1999 until December of 2009, 210 leukapheresis was performed to 50 patients who presented a severe neutropenia (<100/mm^3) due to chemotherapy or hematopoietic stem cells transplantation (HSCT) (allogeneic 53%, autologous 21% and chemotherapy 26%). The population studied was composed for: 24 males and 26 female with an average of age of 31 years (r: 7-56). The hematological neoplasm were 27pts AML, 7pts ALL, 4pts NHL, 3pts CML, 3pts sAA, 2pts Myeloma and 4pts with solid tumors. ABO and Rh compatible healthy donor were injected subcutaneously with 5 ug/colony-stimulating factor (G-CSF) 24 and 8 hs before the Leukapheresis procedure. The product was radiated immediately before the infusion with 30 cGy. Fourth-seven (96%) presented clinical infectious focus, being the most frequently cellulitis, severe mucositis and pneumonia. The rescue microbiological was documented in 31 patients (62%): Gram-negative bacilli 16pts, Gram-positive cocci: 6 pts, and 9 pts with fungi infections. Six-teen pts, (34%) it not was found microbiological rescue. The three remaining patients did not present focus neither germ. *Results.* With an estimate of transfusions to the clinical control of the focus or recovery of neutrophil its was carried out an average of transfusion/patient of 4.2 (r: 1-15); the average of granulocyte per transfusion was of 3, 25×10^{9}L (r: 1 -7-3) Adverse reactions due to the granulocyte transfusion therapy were: one case rush, dyspnea and one case pulmonary infiltrate. Thirty-one from 50 patients analyzed (62%), responded to the GTT, with clinical improvement. According to the causal germ it was observed that the group with Gram-positive resolved the event in the 66% of the cases; 63% of the G-negative and 77% of the patients with fungal infections ,while in the group in which not germ was isolated, 36% was resolved. *Conclusions.* granulocyte transfusion is a feasible procedure for those patients who do not respond to antimicrobial therapy, with a good focus resolution index in patients with severe neutropenia, and microbiological documentation.

1916

VALIDATION OF A COMMERCIAL CRYOPRESERVATION MEDIUM: POST-THAW RECOVERY OF CD34 AND OTHER WBC

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Background. Collection and cryopreservation of autologous stem cells is a routine therapeutic treatment in Lymphomas, multiple myelomas, neuroblastomas, nephroblastomas and other malignant diseases. We validated the commercial freezing medium CryostorTM (CS10, 10% DMSO) in comparison to our routinely used 20% DMSO and 10% human plasma derivate (Biseko^{TM}) in Ringer solution.Aims Aim of the study was to examine whether or not the recovery of viable WBC and CD34$^+$ cells was better with CS10 as compared to our conventional freezing medium.Methods Fourteen aliquots from 7 stem cell collections were 1+1 mixed with either conventional freezing medium or with protein-free CS10, frozen in a controlled rate freezer, and stored in the gas phase over liquid N2. The post-thaw recovery of viable WBC & CD34$^+$ cells was determined immediately after thawing and after 20 to 60 min, by multi-color single-platform flow cytometry. *Results.* The mean recovery (+/- SD) of WBC and CD34$^+$ cells was 67.7 (+/- 52)% and 94,3 (+/- 51)% for the conventional medium, and 72 (+/- 12)% and 99 (+/- 16)% for CS10. The T-test for 2 independent samples and different variances revealed no significant differences between both media for WBC (P=0.786) and for CD34$^+$ cells (P=0.746). As demonstrated by the F-test, however, there were highly significant differences between the variances for both WBC (P=0.00007) and CD34$^+$ cells (P=0.0004). Analyses performed 20 and 60 min after thawing revealed a dramatic cell loss (roughly 90%) in the conventional medium for all cell types, up to complete clotting in 3/10 experiments after 20 min, whereas only between 0% and 30% of cells were lost in CS10, and no clotting occurred. *Conclusions.* We conclude that CS10 has clear advantages over our conventional freezing medium, in terms of cell recovery post-thaw, particularly after 20 to 60 min storage at room temperature. Further data will have to be collected to validate the outcome of hematopoietic regeneration after reinfusion.

1917

CLINICAL USEFULNESS OF WEAK AND PARTIAL D RESEARCH IN BLOOD DONORS AND IN TRANSFUSED PATIENTS

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Background. The D antigen of the Rh system is a mosaic of epitopes. In Europe, about 1% of people carries Rh(D) alleles as weak or partial D. A weak D type results from a quantitative reduction of the Rh(D) antigen, whereas a partial D type indicates a qualitatively altered Rh(D) protein. Individuals, whose cells lack some epitopes of the D mosaic, may make antibody to these epitopes when exposed to a complete D antigen. Many studies have shown that there are more than 30 recognisable epitopes, but the "9 epitopes" model is clinically satisfactory. With the use of selected panels of monoclonal anti-D reagents it is possible to distinguish the red cell categories of partial-D phenotypes. On the other hand, based on serological properties different from a weak D type, a partial D type is also suspected in patients with anti-D in serum or if non-reactive to some reagents. *Aims.* In this study we have examined the reactivity patterns of red blood cells, resulted non reactive to routinely used anti-D sera, with 6 different monoclonal anti-D able to obtain an accurate characterization of Rh(D) antigen. *Methods.* We have analyzed 62 blood samples, collected between 2003 and 2009, from donors and patients typed as "suspected"weak D. In fact these samples resulted Rh-negative using monoclonal and polyclonal anti-D sera, but Rh-positive performing the weak D typing with the column

agglutination test and the Coombs' serum. For final and definitive Rh(D) typing we have used the "ID-partial RhD-typing"(Diamed, Switzerland), a card with 6 microtubes containing polyspecific anti-human globulin within the gel matrix. These sera may differentiate between categories II, IV, V, VI, VII, DFR, DBT, R0Har. Moreover, category I is obsolete and category III red cells react with all anti-D reagents but can be distinguished from normal Rh(D) positive cells by the presence of alloantibody anti-D in the serum. *Results.* Our results show 46 cases of weak D on 62 samples tested. 1 subject was genotypized as D type 3 (associated to IAT positive for anti-D), 9 patients as type V, 1 donor as type VI (associated to Ce) and 5 patients type VII (associated to Ce). *Conclusions.* Considering that the most partial D discovered in the Caucasian population are initially typed as weak D, Rh(D) typing in donors and in patients should be routinely performed using more than two anti-D reagents from different clones. Our report suggests that molecular typing of weak D, respect to serotyping, may offer a more reliable classification, probably relevant to optimize transfusion strategies. Partial DVI remains the most important category to define. Clinically, patients with partial DVI red cells should be treated in the same way as Rh(D) negative individuals both for transfusion purposes an during/after pregnancy with a Rh(D) positive fetus, to prevent the alloimmunization *vs.* the missing epitopes with possible subsequent clinical consequences.

1918

WHY IS POTENTIAL BLOOD DONORS DEFERRED FROM DONATION?

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Background. Voluntary donation is a key feature of blood banking. To ensure the safety of blood transfusions, careful donor selection is important. Although new approaches to blood safety have dramatically reduced the risks for infectious contamination of blood components, the quality and the availability of blood components depend on the willingness to donate and the reliability of the information given by the donors about their own health, including risk behavior. Donor education is an important part of this effort, and this is highlighted in the European Blood Directive. As donors who are deferred by the blood bank will be less motivated to return for donation, it is important to reduce the number of deferrals. However little is actually known about the reasons for why regular donors turn up in the blood bank for donation without fulfilling the donation requirements. *Aims.* The aim of the present study was to investigate the reasons for deferral of registered donors coming to the blood bank for donation; in order to identify areas of importance for donor education - as these deferrals potentially could be avoided by better donor comprehension.

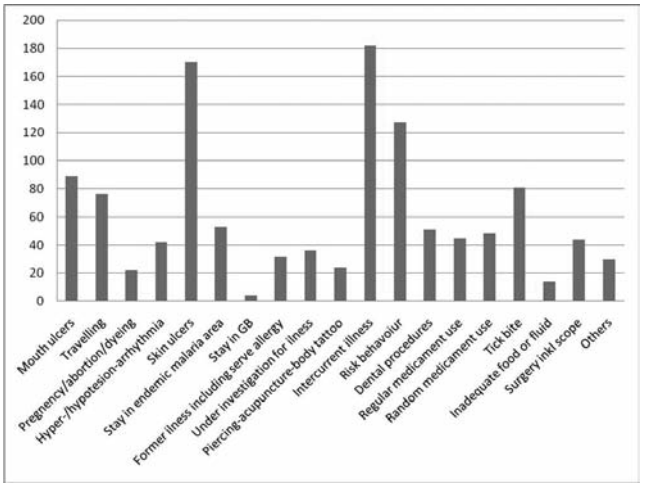

Figure 1. Reasons for deferral of 1 163 regular blood donors.

Methods. Data were collected from all blood donors who came to the Blood bank at Haukeland University Hospital, in the period between June 15, 2008 and December 15, 2009. All blood donors answered a questionnaire form about on health condition and potential risk behaviour. Results were collected and present after standard statistical models. *Results.* 1 163 of the 29 787 regular donors, who showed up for donation, were deferred (3.9%). The results demonstrate that there are multiple reasons for denying a potential donor to give blood, as summarized in Figure 1. The main reasons were intercurrent illness (n=182), skin ulcers (n=170) and risk behaviour (n=127). The underlying intercurrent illnesses were classified according to aetiology or organ system affected. The most common reasons were airway infections (n=62), gastrointestinal symptoms (n=24) and worsening of season allergy (n=10), in addition to a large group of unknown or unclassified reasons (n=58). *Summary/Conclusions.* Various measures are taken to ensure the safety of the blood supply, but donor selection and education are still the most important strategies we have to avoid potential complications to transfusion. It is important that potential donors must fill in honestly a questionnaire designed to identify specific risk factors for infectious conditions. In a community, intercurrent disease, skin ulcers and potential risk behavior are the most frequent reasons for deferral of regular donors. Strategized effort on donor education is needed, as "failure to donate"reduce donor motivation.

1919

PREVALENCE OF CYTOMEGALOVIRUS(CMV) AND EPSTEIN-BARR VIRUS(EBV) IN GREEK MULTIPLY TRANSFUSED PATIENTS WITH THALASSEMIA

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Background. Cytomegalovirus (CMV) and Epstein-Barr virus(EBV) maintain life-long latent persistence in the majority of the adult population,including blood donors. The potential risk of CMV and EBV transmission by blood components is not well established. *Aim.* The objective of this study was to determine the prevalence of CMV and EBV among thalassemic patients and healthy blood donors of Thessaly prefecture in order to evaluate the possibility of CMV and EBV blood-born infection in Greek population. *Methods.* Fifty five(55) multiply transfused patients with thalassemia and haemoglobinopathy were included in the study (28 men, 27 women,median age: 28). The majority had received multiple blood transfusions,from 12-36 per year. Serologically, 18 of 55 patients (32,72%) were HCV^+. The control group consisted of two hundred eighteen (218) randomly selected blood donors of central Greece (183 men, 35 women,median age: 38). DNA was extracted from whole blood specimens by the automated Magtration system 12GC plus (magnetic particles technology). Presence of CMV and EBV genome copies was evaluated using single-virus quantitative real time polymerase chain reaction (qrt-pcr) assays by Nanogen Advanced Diagnostics. Amplification reactions were specific for the EBNA-1 and MIEA regions of EBV and CMV respectively. Cellular target for the beta globin gene was used to quantify cell-equivalent DNA. *Results.* CMV DNA was not detected in any patient or donor sample. In contrast 8 of 55 blood specimens among thalassemic patients (14,545%) and 42 of 218 samples among blood donors (19,26%) had detectable EBV DNA. Seropositivity for HCV co-existed in 5 of 8 EBV DNA^+ patients (62,5%). *Conclusions.* No significant difference was found in EBV DNA prevalence between the two groups (P>0.05). There are no indications for EBV transmission through transfusion in Greek population. It appears that leukoreduction of blood units provides some benefit in thalassemic patients.

1920

HEMOSURVEILLANCE OF DONORS: REPORT OF EARLY AND LATE COMPLICATIONS AFTER A BLOOD DONATION OF 450 ML

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Background. Hemovigilance is a set of surveillance procedure covering the complete process from blood donation to transfusion. According to European and Italian transfusion legislations, the monitoring of

reactions and/or complications following donation is an integral part of hemovigilance. In fact, the report of reactions at the phlebotomy site and of observation of complication during or after blood letting is a standard part of donation documentation. On the contrary, data about late reactions, after leaving the blood bank, are not always available. *Aims.* The aim of this study was to analyse rate and types of complications and reactions reported by donors one hour after a donation of whole blood. *Methods.* In order to obtain a correct evaluation of these post-donation events, we have invited all donors, in a period of one year, to remain under observation for 1 hour after donation in our Transfusion Centre. Collected data were statistically analysed. *Results.* We have observed 5.487 people who donated whole blood (450±10 mL) and 918 (16,7%) adverse events have been registered. The most common findings have been 411 cases (7,5%) of bruise and/or haematoma; in particular we have observed 184 cases (45%) with a reported diameter of haematoma <1 cm, 161 cases (39%) with a diameter of 1-3 cm, 45 cases (11%) of 3-5 cm and 21 cases (5%) > 5 cm. Arm nerve injuries (soreness, sensory changes, numbness, tingling) have occurred in 162 donors (2,9%). 83 donors (1,5%) have reported local irritation or/and allergic reaction in shape of the plaster. The most common systemic reactions (4,7%) have been fatigue, vasovagal symptoms and nausea and vomiting (262 cases: 187/4.099 = 4,5% of men and 75/1.388 = 5,4% of women). Three of donors had to seek medical care, one of them has been hospitalized. Periodic donors, of course, have developed fewer reactions than subjects at their first donation and women have showed fewer reactions than men. The frequency of systemic reactions has been dependent on weight of donors, with the highest frequency in the group of 50/60 Kg. *Conclusions.* In this study, the frequency of observed complications and reactions, in our opinion, is very high, but in according with available data of other Italian Transfusion Centre. Some reactions and complications are honestly predictable and avoidable, for this reason a careful management of donors need. Moreover, in order to guarantee a major safety for donors, the participation of all staff in educational and training program is also important.

1921

PLATELET TRANSFUSION AND INCIDENCE OF FEBRILE NON-HAEMOLYTIC TRANSFUSION REACTIONS (FNHTR) IN PAEDIATRIC PATIENTS: A COMPARISON BETWEEN PLASMA REDUCTION AND LEUKODEPLETION OF PLATELET CONCENTRATES

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Background. Transfusion of blood products is often necessary and beneficial for critically ill patients. Nevertheless, transfusion can lead to serious adverse effects; one of these is the febrile non-haemolytic transfusion reaction (FNHTR), that, on the basis of international data, complicates 2 to 37 percent of transfusions of random platelet concentrates (obtained from a single donor platelet-rich-plasma) in adults. On the other hand, platelet concentrates are frequently transfused in paediatric patients affected with a severe thrombocytopenia too, but the incidence of FNHTRs in these patients is not well known. *Aims.* In this study we have evaluated the incidence of FNHTRs after platelet transfusion in children. In addition, in order to identify a technical procedure able in reducing this incidence, we have also compared the results obtained from 3 different hemocomponents: 1) the "standard"platelet concentrate, obtained from a single donor platelet-rich-plasma; 2) a platelet hyper concentrate, characterized by reduction of plasma volume after storage; 3) a filtered platelet concentrate with leukodepletion before storage. *Methods.* Children from 1 until 14 years of age were eligible for this study, independently from their pathology. For the first aim of this study, we have retrospectively analyzed the platelet transfusions, using unmodified whole-blood-derived platelet concentrates, performed in a period of six months. For the second aim, in a first period of three months we have transfused platelet concentrates submitted to centrifugation and reduction of the plasma volume just before transfusion; while in a second period of three months we have used prestorage WBC-reduced hemocomponents, but the platelet concentrates were stored for a maximum of two days. Signs and symptoms characteristic of a FNHTR during, immediately after and 2 hours following transfusion were registered. *Results.* In the considering period, 115 platelet transfusions were administered to 77 children; in particular 57 "standard" platelet concentrates were transfused to 35 children enrolled in the study, 29 removed plasma hemocomponents were given to 20 little patients, while 29 leukodepleted platelets were administered to 22 subjects. A FNHTR was observed in a total of 11 cases on 115 transfusions, with an incidence of 9.6%, a value not significantly different from the one registered in adults. Analyzing the three kind of transfusions, no statistical significant differences were registered (P>0.05), in fact we have observed a FNHTR incidence of 12.3% (7 on 57 transfusions) in the first group of patients, of 6.9% (2 on 29) with removed plasma platelets and of 6.9% (2 on 29) with WBC-reduced platelets. In addition, allergic reactions occurred respectively in 8.8% (5 on 57), 3.4% (1 on 29) and 6.9% (2 on 29) of platelet transfusions. *Conclusions.* Even if no statistical evidence exists, FNHTRs may be less common among paediatric recipients of platelet transfusions than in adults. Moreover, in our study, a lower frequency of FNHTRs and allergic reactions was observed using platelet concentrates treated with post-storage plasma removal and/or pre-storage leukodepletion, but our record of cases is yet too much little to produce definitive conclusions.

1922

DOCUMENTATION OF KNOWLEDGE AND BEHAVIOR OF THE YOUTHFUL POPULATION CONCERNING HEPATITIS B AND AIDS

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Background-Aim. To scrutinize and evaluate the knowledge of young people and to document their behavior, concerning Hepatitis B and AIDS. *Material - Method.* A total of 521 answers were studied, of young people who responded to an anonymous closed type questionnaire. 215 were high school students 15-18 years of age, 120 were Technological Educational Institution (T.E.I.) students 18-25 years of age, and 186 between the ages 25-35. Their knowledge level and behavior was scrutinized. The data process was performed with SPSS v.12.0. *Results.* The knowledge of the T.E.I. students, concerning the ways of transmission and prevention of Hepatitis B were satisfactory at 78%, while for AIDS at 97%. The group of 25-35 follows with percentages 72% and 96%, while high school students are last, presenting sufficient knowledge about Hepatitis B and AIDS in 62% and 95% correspondingly. Concerning AIDS medication, 26% of T.E.I. students have an elemental knowledge, 13% of the young adults and 10% of the high school students. 76% of T.E.I. students use a condom, 65% of the teenagers; while in young adults only 23% uses a condom (75% of them claim that they have a monogamous relationship). Moreover, 11% of the T.E.I. students and 19% of the adults claimed that they have been tested for AIDS, either after a dangerous sexual contact, or with some other opportunity (e.g. volunteer blood donor). Finally, 26% of high school students, 22% of T.E.I. students and 14 % of the adults has been vaccinated against Hepatitis B. The main source of information for this diseases, were the MEDIA in 70%, the family in 18% and the school in 12%. A total of 81% requests more information about AIDS and 93% about Hepatitis B. *Conclusions.* 1) The students knowledge is the most sufficient, followed by that of young adults, while the teenager's knowledge is lacking, a fact that can be attributed to the laxity of information campaigns in schools the last years. 2) The awareness in general concerning hepatitis is lacking compared to AIDS, a fact that needs to be dealt with. 3) Even though the knowledge of the population is satisfactory in general, a big percentage requests more information campaigns, which is rather comforting. It is pertinent, that this will be done in schools, which are lacking in knowledge, given the fact that an important percentage of students, especially of young age, demonstrate impermissible negligence concerning protection.

1923

A HTLV LOOKBACK STUDY IN KOREA

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Background. Human T-cell lymphotrophic virus (HTLV) is known to be the significant etiologic retrovirus of adult T-cell leukemia(ATL) and HTLV-associated myelopathy(HAM). Blood transfusion is known to be the major source of HTLV infection, but few studies were done on risk of transmission -associated infection. *Aims.* The sero-conversion rate of HTLV via blood transfusion in Korea has not been identified yet. This study is aimed to identify the sero-conversion rate in recipients of blood

provided from the infected donors with HTLV in Korea. *Methods.* One hundred fifty three recipients of the blood components from 34 index donors with HTLV were recognized through an investigation. A retrospective study to investigate whether transfusion-transmitted HTLV infection occurred in the recipients were carried out by the Korean Centers for Disease Control and Prevention (KCDC) between 2008 and 2009. Among the recipients, 39 persons agreed with informed consent for this study. Laboratory tests and epidemiological studies of the participants as well as tests for all stored samples of the index donors were fulfilled. For the laboratory tests, line immune assay, western blot and PCR with HTLV were applied. *Results.* It was found out that 59 recipients were alive and 74 were deceased among the 153 recipients. One person was not transfused and no data were available for the rest 19 people. Participants of the study were 39 out of 59 who agreed with informed consent, whereas 20 recipients refused. Fifteen recipients (38.5%) by 13 index donors in total, were identified as HTLV positive status. There were no ATL and/or HAM cases developed in the positive cases. Besides, the test results of the stored samples were all positive with HTLV. *Conclusions.* A targeted lookback study to trace 153 recipients by 34 index donors with HTLV was carried out in Korea. In total, 15 recipients out of 39(38.5%) were resulted with HTLV positive status. There was no case of ATL and/or HAM cases developed in the positive cases.

1924

EXSANGUINO-TRANSFUSION AND RECONSTITUTION OF WHOLE BLOOD: AN OPERATIVE PURPOSE FOR STANDARDIZATION

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Background. Italian Scientific Societies of Transfusion Medicine (SIMTI) and Neonatology (SIN) have collaborated to the drafting of a document named "Recommendations for transfusion therapy in the neonatal period"because an observed difference between every Italian Hospital in the transfusion therapy reserved to anaemic newborns. *Aims.* In order to standardize the behavior between all Transfusion Centers in our Region, we developed a supplementary operative technical proposal for preparation of the reconstituted whole blood (RWB) and to establish its final characteristics. Our aim is to largely promote this document. *Methods.* According to Recommendations, RWB must be produced in Blood Transfusion Service through the combination of Erythrocyte Concentrate (EC) and Fresh Frozen Plasma (FFP). The used EC should be: preferably of group "0" or compatible with the newborn from an immunohematologic point of view; leukocyte-depleted (and, consequently, CMV-safe); fresh (<7 days from collection) and free of additive solutions. The used FFP should be: preferably of group "AB"or compatible with the newborn from an immunohematologic point of view; treated for viral inactivation. *Results.* Our protocol recommends the use of Erythrocyte-apheresis (from multicomponent donation) and Plasma-Safe (with industrial or home-made viral inactivation). The operating procedure involves the following steps: 1) compatibility assessment and choice of units; 2) in all cases, irradiation of EC; 3) centrifugation and hyper-concentration of EC; 4) sterile connection to a sterile transfer bag and removal of supernatant (= additive solutions); 5) determination of the weight of the remaining EC; 6) sterile connection of EC to FFP and transfer of an equivalent weight of plasma (in order to obtain a Hct of about 50%). The whole procedure must be performed by ensuring total asepsis. *Conclusions.* Multicomponent donation ensures a high efficiency leukocyte-depletion due to apheresis and successive filtration; the partial washing of EC during collection removes the contaminant plasma; at the end, hyper-concentration removes the additive (preservative and anti-coagulant) solutions. On the other hand, industrial Plasma-Safe provides a standardized amount of coagulation factors and guarantees a sterility to a larger amount of viruses and bacterial contaminants. In conclusion, standardization of the procedure and of the final product is indispensable for a correct clinical application and evaluation of therapeutic results.

1925

RISK OF ANTI-HLA ALLOIMMUNIZATION DUE TO RESIDUAL LEUKOCYTES IN PLASMA UNITS

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Background. In literature, it has been reported that viable leukocytes (WBC) are present in Fresh Frozen Plasma (FFP), even following freeze-thaw. This observation has implications for possible transfusion-related adverse effects: in fact the presence of WBC in FFC may, even if rarely, cause an anti-HLA alloimmunization and, theoretically, a transfusion-associated graft-versus-host disease (TA-GVHD); moreover leukocyte cytokines may cause febrile reactions. *Aims.* We have compared WBC levels in plasma prepared by three collection methods used at our Transfusion Medicine Units. *Methods.* Residual WBC were quantified by fluorocromatic methods (ideated by Borzini *et al.*.) in fresh (unfrozen) plasma prepared from: 71 whole blood units, 45 filtered on line blood units and 59 plasmapheresis collected by Fresenius and Haemonetics cellular separators. *Results.* Mean WBC levels in 71 plasma units produced by whole blood, with removing of buffy-coat and producing a platelet concentrate, is 47.8 cells/μL (ranged 28.6-88.5), considering the mean volume of these units = 206 mL (range 188-234), in these FFP units there are 9.8×10^6 WBC (range 5.9-18.2). Mean WBC levels in 45 plasma units produced after leukodepletion by filtration of whole blood, without producing a platelet concentrate, is 18.2 cells/μL (ranged 8.4-46.9), considering the mean volume of these units = 244 mL (range 208-256), in these FFP units there are 4.4×10^6 WBC (range 2.0-11.4). Mean WBC levels in 59 plasmapheresis is 1.6 cells/μL (ranged 0.5-5.0), considering the mean volume of these units = 388 mL (range 339-414), in these FFP units there are 0.6×10^6 WBC (range 0.2-1.9). *Conclusions.* Depending on the method of preparation, the number of whole WBC in fresh unfrozen plasma units ranged from a minimum of 0.2 to a maximum of 18.2×10^6 per component. Additional studies are needed to determine the significance of these results with respect to the risks for TA-GVHD and WBC alloimmunization.

1926

MECHANISMS AND CLINICAL IMPORTACE OF STATIN DROP AFTER EXTRACORPOREAL LDL-CHOLESTEROL ELIMINATION

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Background. Therapeutic hemapheresis contributed to the therapy of some disorders with new possibilities. Using our statin analysis method, it was possible to uncover a significant drop in statin levels after extracorporeal LDL-cholesterol elimination (EChE) in severe familial hypercholesterolemia (FH). The purpose of this work was to identify the mechanism underlying this drop and its clinical significance as well as to propose measures to optimize a pharmacotherapeutical regimen that can prevent the loss of statins. *Methods.* Ultra-high performance liquid chromatography connected to a triple quadrupole tandem mass spectrometry (MS/MS) system was used. *Patients.* One hundred and seventy samples were analyzed (85 pairs) from a group of long-term treated patients (3-12 years of treatment) with severe familial hypercholesterolemia (FH) (12 patients) who were treated regularly by LDL-apheresis (immunoadsorption) or hemorheopheresis (cascade filtration). *Results.* After EChE, the levels of statins and their metabolites decreased (atorvastatin levels before and after LDL-apheresis were 8.83 and 3.46 nmol/L, respectively, and before and after hemorheopheresis were 37.02 and 18.94 nmol/L, respectively). A novel finding was a specific loss of statins in washing fluids (concentrations of atorvastatin in washing liquids for LDL-apheresis and hemorheopheresis were 0.28 and 3.04 nmol/L, respectively) and filters (11.07 nmol/L atorvastatin lost in filters during hemorheopheresis). To prevent a substantial loss of statin concentrations, the pharmacotherapeutical regimen should be changed to have a longer time interval between the dose of statins and EChE (15 hours). *Conclusions.* A specific loss of statins was found in adsorbent capsules and filters, especially during hemorheopheresis. The decrease in statin levels can be prevented by the suggested dosage scheme.

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1927

THERAPEUTIC ERYTHROCYTAPHERESIS (TE) IS THE FIRST LINE THERAPY OF HEREDITARY HEMOCHROMATOSIS (HH)

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Background. Hereditary hemochromatosis (HH) is a common genetic disorder in which the iron absorption is increased, resulting in excessive iron overload in parenchymal cells with organ failure, especially with regard to the liver, pancreas, heart, joints and pituitary gland. The gene involved in the most common form of HH was called HFE and the most common mutations C282Y and H63D. The therapy is based on the removal of the excess of body iron by removing blood through phlebotomy. More recently mechanical removal of erythrocytes through therapeutic erythrocytapheresis (TE) has become a new therapeutic modality. *Aims.* In this study we have evaluated efficiency and safety of TE respect to phlebotomy to decrease iron overload in patients with hereditary hemochromatosis. *Methods.* Between 2008 and 2009, we have treated 42 patients affected by HH: 23 patients aging 31 to 78 years were treated with TE while 19 aging 43 to 73 years with phlebotomy. All patients suffered from increased aminotransferase, moreover in the TE group 4 patients suffered from heart failure and 5 patients suffered from liver cirrhosis. TE procedures were performed using Fresenius Comtec Kit PL-1. *Results.* In the considered period we have performed 64 TE: the mean value of removed erythrocytes in each procedure varied between 330 and 624 mL per individual patient. Most of the patients best tolerated an interval period of two weeks. The initial ferritin values varied between 350 to 3120 while at the end of the treatment in the TE group the serum ferritin levels varied between 22 and 88 ng/mL. The total number of treatments needed in the TE group varied between 3 and 15. The duration of treatment was between 1 and 10 months. In the phlebotomy group the serum ferritin levels varied between 36 and 76 ng/mL. The total number of treatments varied between 9 and 22 months with a total removed volume of 400-450 mL of whole blood. in the 75% of cases we have observed the normalization of liver enzymes. *Conclusions.* The results of this study indicate that TE compared to phlebotomy in the therapy of HH patients was both safe and highly efficient to remove iron excess, in fact both the number of procedures as well as the duration of the therapy was significantly reduced. An isovolemic method of therapy is therefore potentially safer than phlebotomy with its related induced hypovolemia, especially with respect to older and/or cardiovascular unstable patients. TE also preserves the valuable blood components of the patient, such as plasma proteins, platelets, clotting factors and leucocytes, potentially relevant to patients with hypoproteinemia and/or thrombocytopenia. During a TE procedure the patient receives compensation for the removed volume by saline or protein solutions, which makes this approach particularly viable for HH patients with severe cardiac disease. TE appears to be a better tolerated type of treatment applicable to a wider range of patients, in fact no adverse side-effects were observed in patients treated with TE.

1928

DEVELOPING A PREDICTIVE MODEL FOR RED BLOOD CELL TRANSFUSION REQUIREMENT FOR ACUTE LEUKEMIA PATIENTS

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Background. Faced with the ongoing reduction in the number of blood donors, the clinical hematologist must often ask patient families for help in finding suitable donors. Another benefit of the predictive model is an improved communication with the blood bank, allowing the clinical hematologist to order the appropriate amount of blood units necessary for the whole duration of the aplasia. *Aims.* Creating a predictive model for red blood cell transfusion requirement in a patient with acute leukemia (mieloblastic and limphoblastic) patients in aplasia following chemotherapy. *Methods.* Study type: analytical, cohort, retrospective. We studied 246 patients with acute leukemia admitted in the Clinic of Hematology in Cluj-Napoca during 1995-2008 and treated according to international protocols (exclusion of palliative care therapy, deceased or transferred patients). 860 aplasia episodes secondary to chemotherapy were included in the study. All the patients signed the informed consent. The study has the agreement of Ethic Committee of "Iuliu Hatieganu" Medicine and Pharmacy University Cluj-Napoca, Romania. Statistical analysis was performed using a linear model (multiple regression). Independent variables used in setting out the predictive models were: age, gender, morphologic type of leukemia, bone marrow blast infiltrate, disease stage at the beginning of chemotherapy regimen (diagnosis, complete remission, partial remission, refractory disease, no response), chemotherapy regimen number, medullar erythrocyte line (normal, low, dysplastic), chemotherapy type (high-dose or standard-dose), hemoglobin levels at chemotherapy start and at the end of the aplastic period, hemorrhagic episodes during the next aplastic period, hemolytic transfusion reaction, compatibility testing type for transfused blood (classic or gel migration methods). Finding the best regression model was based on univariate analysis of all risk factors (chi-square tests). Multicollinearity was excluded when heavily correlated pairs of independent variables were found. Statistical analysis was performed using SPSS, Statistica and Excel. *Results.* The number of red blood cell units necessary to be transfused to an acute leukemia patient in an aplastic phase following chemotherapy can be estimated using the following equation: Number of blood units = 7.01-0.35 × Starting Hb + 0.77× Diagnosis - 0.91 × Partial remission - 1.01 × Complete remission + 1.21× Minor hemorrhages + 3.41 × Major hemorrhages + 1.31× Haemolysis. Number of blood units is the amount of whole blood and/or red blood cells concentrate units necessary. Independent variables Diagnosis, Partial remission and Complete remission (disease stage at chemotherapy), Minor hemorrhages and Major hemorrhages are assigned 1 if present and 0 if absent. Minor hemorrhages were represented by low quantity epistaxis, bleeding gums, purpura, etc. Major hemorrhages were represented by menorrhagia, haematemesis, melena, etc. This model has statistical significance (ANOVA test, $P<0.05$, statistically significant) therefore it can be generalized from the study group to the whole population. *Conclusions.* The variables representing the best predictors of red blood cells blood units number transfused to an acute leukemia patient in an aplastic phase following chemotherapy were: hemoglobin levels at chemotherapy start, disease stage at the beginning of chemotherapy regimen, hemorrhagic episodes during the next aplastic period and hemolytic transfusion reactions.

1929

APHERESIS ACTIVITIES IN A BELGIAN UNIVERSITY HOSPITAL

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Background. Apheresis is a potent tool in both collecting and removing blood components. Using centrifugation-based devices both plasma and cellular components can be efficiently separated. *Aim.* We undertook a retrospective analysis of all centrifugation-based apheresis-procedures accomplished at the University Hospitals of Leuven from Januari 2006 till September 2009, aiming to obtain information about single center activity range, indications and outcome. *Methods.* Medical apheresis-records of all patients and donors subjected to therapeutic plasma exchange or cytapheresis were used for this retrospective observational study. *Results.* During this period 3877 procedures were performed, with therapeutic plasma exchange (TPE) being executed most frequently (77.4%). Neurological indications accounted for 58% of the total TPE activities, whereas 34% of the indications were hematological. Neurological indications included myasthenia gravis (37.9%), (monoclonal gammopathy-associated) polyneuropathy (17.7%), chronic inflammatory demyelinating polyneuropathy (8.1%), stiff-person syndrome (8.1%) and multiple sclerosis (7.3%). Thrombotic thrombocytopenic purpura/haemolytic uremic syndrome was the most frequent haematological indication (67.1%), followed by hyperviscosity syndromes (19.1%). Based on the AABB/ASFA guidelines for TPE, which classifies the indications into four categories ranging from standard and acceptable therapy (I) to no therapeutic benefit (IV), we calculated the proportions of indications in our center for these different categories. 142 (66.4%) indications were classified as category I/II, whereas category III/IV/NR/others included 72 indications (33.6%). Besides TPE, 22.6% of the procedures were based on cytapheresis: autologous hematopoietic progenitor cell collection (HPC-A) (33.8%), therapeutic dentritic cells (33.1%), allogeneic HCP-A (21.0%), cell depletion (9.9%), therapeutic T cells (1.7%) and extracorporal photopheresis (0.5%). Concerning HPC-A we performed an outcome analysis based on the fact whether a previous determined target (desired number of CD34+ cells/kg) had been reached. In this analysis only HPC-A procedures

between 1th January 2008 and 1 October 2009 were included. During this period 155 HPC-A procedures (90 autologous and 65 allogeneic) were performed. Target was reached in 119 (76.8%) of these collections. *Conclusion.* We report a retrospective analysis of all centrifugation-based apheresis-procedures performed in a Belgian University hospital during a 44 month period. Results of indications seems to be close to existing guidelines for less rare disorders, although the relative high number of requests for type III/IV/NR/other categories may reflect the amount of referrals of difficult-to-treat diseases to a university center, emphasizing the need for clinical trials in these disorders. Outcome analysis in HPC-A, although limited to the observation whether a target had been reached or not, seems to be comparable to similar data in the literature.

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A SINGLE INSTITUTION EXPERIENCE WITH NOVOSEVEN USE

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Background. The recombinant activated factor VII (rFVIIa NovoSeven, Novonordisk, Denmark) was developed and approved for the treatment and prevention of bleeding episodes in patients with a history of congenital hemophilia, acquired hemophilia, congenital deficiency of F-VII and Glanzmann thrombasthenia. Currently, NovoSeven is being administrated as a drug for compassionate use in cases of life-threatening bleeding. *Objective.* To analyze retrospectively the indications for administration of NovoSeven in a single center. *Patients and methods.* The hospital pharmacy database was used to identify patients in which NovoSeven was administered during the period 2006-2009. A total of 22 patients (16 M/6 F; median age 54 years) were included in the analysis. rFVIIa was administered for different reasons: surgical prophylaxis in congenital FVII deficiency (n=1), acute promyelocytic leukemia with pulmonary hemorrhage (n=1), renal transplant hemorrhage (n=1), multiple trauma (n=4), upper gastrointestinal bleeding (n=4), internal bleeding (n=4), renal contusion (n=1), subdural hematoma (n=1), basal ganglia hematoma (n=1), subarachnoid hemorrhage (n=1), intraventricular hemorrhage (n=1), compartment syndrome (n=1) and ruptured abdominal aneurysm (n=1). A total of 48 doses were administered, and the average dose per patient was 2.18 units. In addition to NovoSeven, patients received on average 13.5 packed red blood cells, 5.6 units of fresh frozen plasma and 3.14 pools of platelets. *Results.* The average prothrombine time (PT) before administration of rFVIIa was 37.71% and 105.54% after NovoSeven infusion. Mean INR before administration of rFVIIa was 2.72 and subsequently 1.21. Nine out of 22 patients (41%) died, being the causes: hypovolemic shock (n=4), underlying medical conditions (n=5); one was transferred to another ICU institution and 12 (55 %) developed favorably. Two patients presented thromboembolic events following the administration of rFVIIa (thrombosis of arteriovenous fistula in a patient with chronic renal failure and cardioembolic stroke). *Conclusions.* NovoSeven was administered only in one patient according to the approved indications. More studies are needed to demonstrate the usefulness of rFVIIa in bleeding events without licensed indications. Consensus guidelines for NovoSeven use in clinical practice are needed.

1931

ENZYME REPLACEMENT THERAPY OF EGYPTIAN CHILDREN WITH GAUCHER DISEASE: SINGLE CENTER EXPERIENCE

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Background. Gaucher disease (GD) is the most frequently encountered lysosomal storage disease caused by inborn defects of the membrane-bound lysosomal enzyme, acid β- glucosidase . This defective activity causes accumulation of glucocerebroside in the lysosomes of cells derived from the monocyte/ macrophage lineage. Glucocerebroside-engorged cells, termed Gaucher cells, infiltrate various organs, leading to multisystem abnormalities *Aim of study.* to present a single center experience in the diagnosis and management of Gaucher disease in Egyptian children with emphasis on phenotype-genotype relationship. *Patients and methods.* The study included 48 patients with Gaucher disease attending the Children's hospital, Ain Shams University, Cairo, Egypt, from June 1995 to December, 2006. Patients were diagnosed by low β- glucosidase enzyme activity. Enzyme replacement therapy was started in 1997 using the placenta derived aglucerase (ceredase) at low dose regimen (15 U / Kg / 2 weeks); recombinant enzyme imiglucerase (cerezyme) therapy started in March 1999 in a dose of 20-30U / Kg / month, divided into 4 equal weekly doses. In 1999 cerezyme was administered in a dose regimen of 60 U / Kg / 2 weeks. *Results.* Patients ages at presentation ranged from 37 days to 17 years with mean age of 2.54± 3.8 years. They included 10 patients with type I, 6 with type II and 26 with type III Gaucher disease. Clinical and laboratory follow up for at least 24 months revealed a significant increase in weight and height, significant reduction in the liver and spleen measurements, normalization of PT and PTT, and significant increase in haemoglobin level and platelets counts. Individual variations were observed in patients' response to ERT regarding neurological manifestations: while there was mild improvement in motor development and variable improvement of dysphagia on ERT, strabismus and ophthalmoplegia did not. Bone manifestations showed very slow improvement on ERT; but all patients demonstrated improvement in quality of life in an average period of 6 months. Plasma chitotriosidase levels showed significant decrease after ERT assay .Mutation analysis of 23 patients (14 type III, 8 type I and one type II) showed that 56.4% were homozygous L444P (11 patients type III and 2 patients type I). *Conclusions.* Since most of Egyptian children with GD have type III disease and L444P/L444P genotype, a minimum dose of 60U/kg/2 weeks should be maintained until adulthood. Higher doses started at an early age may delay the progression of neurological symptoms. Pulmonary involvement is not rare in Egyptian patients and may respond to dose increase or dose fractionation. Cardiovascular and renal symptoms should be further studied in our population.

1932

PANCYTOPENIA SECONDARY TO CHRONIC HEMOPHAGOCYTOSIS IN ASSOCIATION WITH WEBER CHRISTIAN DISEASE

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Introduction. we report a case of chronic hemophagocytosis in a patient with Weber-Christian disease. A forty-two -year-old female suffered, since her childhood, from pain and inflammation at hands, ears and cheeks skin, especially in cold weather and fever episodes that lasted 2 or 3 days. Twelve years ago pancytopenia was detected with the following blood counts: white cells $1,2\times10^9$/L, neutrophils $0,6\times10^9$/L, Hb 109 g/L, VCM 76 fL and platelets 104×10^9/L. For the last six years she has been monitored in our hospital. Hepatoesplenomegaly was always 1cm below the costal margin, lymphoid nodes were never detected at the physical examination or CT-scans. Pancytopenia didn't change and several tests showed high levels of LDH (700U/L) and consistently low levels of haptoglobin (<7) and low retyculocite counts (0.7%). Blood smear was normal. Studies for immune hemolysis, thalassemia, hemoglobinopathies, enzymopathies, HPN and red blood membrane diseases were negative. Ferritin 750 μg/L, folic acid and B12 vitamin had normal values. Three bone marrow studies carried out in different moments of the clinical evolution, only showed hystiocitic hiperplasia with outstanding hemophagocytosis of blood cells, neutrophils and platelets. Infection or lymphoma were not documented. Bone marrow cytogenetics was normal. Fanconi anemia was excluded in 2006 by chromosome breakage test. The study of peripheral blood and bone marrow markers was normal. Cytoplasmic expression of perforin was preserved. Rheumatoid factor was 39 UI/mm in 2004, increasing later to 244UI/mm, but the patient never met diagnostic cryterias for rheumatoid arthritis or LES. A skin punch was not diagnostic. Altought transaminases doubled the normal value, the liver biopsy only showed changes due to systemic disease. In January 2009 she noticed right eye exoftalm and retroocular mass biopsy showed Weber-Christian panniculitis. Alfa-1-antitripsin was normal and C1q determination was above the normal value 428 mg/L (n.v 100-255 mg/L). When she was receiving prednisone treatment for the retro-ocular panniculitis she developed fever and deep pancytopenia with neutrophils $0,1\times10^9$/L, platelets 15×10^9/mL, increased D-dimer 571 ng/mL, ferritin 2.184 μg/L, triglicerids 219mg/dL as well as EBV copy number to1350/mL. Bone marrow biopsy was similar to previous biopsies. The patient improved when prednisone was tapered and i.v. gammaglobulin was prescribed. The patient felt well, pancytopenia improved to levels of the six previous years and EBV copy number decreased to normal values. Cyclosporine treatment was added to the low dose of prednisone, but her chronic moderate pancytopenia never reverted to complete

response. Five months later, while in this treatment, she suffered colon perforation secondary to a new panniculitis episode. Prednisone and cyclosporine were stopped after 9 months of treatment, now she has started with Thalidomide. *Conclusions.* there are three remarkable points in this case: 1) It is well known the association of hemophagocytosis with rheumatoid arthritis and LES, nevertheless its association with Weber Christian disease is rare. 2) Hemophagocytic syndrome is frequently observed as an acute and serious disease, while this is a well documented case of chronic hemophagocytosis. 3) Hemophagocytic syndrome worsened when the number of EBV copies increased during prednisone treatment for retro-ocular panniculitis.

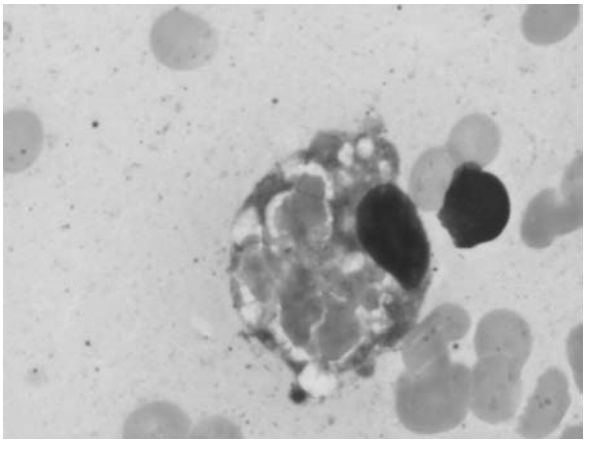

Figure.

1933

REGIONAL VARIATIONS IN GAUCHER DISEASE PRESENTATION: DATA FROM THE ICGG GAUCHER REGISTRY

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Background. Physicians worldwide participate in the International Collaborative Gaucher Group (ICGG) Gaucher Registry, which currently includes data on over 5,700 patients. *Aim.* To analyze data in the ICGG database by geographic region in order to identify regional disease or treatment variations. *Methods.* In 2009, the Gaucher Registry contained patient data from the following regions: North America (n=2108); Europe (n=1477); Middle East/Africa (n= 986); Latin America (n=901); and Asia-Pacific (n=238). Descriptive statistics were provided for demographic and clinical characteristics of Gaucher disease. *Results.* North America and Latin America had the highest proportion of patients with type 1 non-neuronopathic Gaucher disease (96%, 95%, respectively). Asia-Pacific, Europe, and Middle East/Africa had larger percentages (10%, 14%, 16%, respectively) of patients with type 3 neuronopathic Gaucher disease compared to other regions. Asia-Pacific, Europe, Middle East/Africa also had the highest reported frequency of the L444P/L444P genotype (20%, 9%, 11%, respectively) compared with North America and Latin America. Over 77% of patients with Gaucher disease in North America, Europe, Middle East/Africa and Latin America carried at least 1 copy of the N370S mutation. In Asia-Pacific, there were fewer patients with the N370S mutation, with 53% of patients having at least one copy of the L444P mutation. Patients in Asia-Pacific were diagnosed at a younger median age (7yr) than patients in Latin America (11yr), Europe (16yr), Middle East/Africa (10yr) and North America (18yr). *Summary/Conclusions.* Regional differences in ICGG Gaucher Registry data are indicated by the predominance of the N370S mutation, typically associated with the non-neuropathic form of Gaucher disease, in North America, Europe, Middle East/Africa and Latin America. The L444P mutation, which is typically associated with the neuronopathic form of Gaucher disease, was more common in the Asia-Pacific region.

1934

QUANTITATICATION OF MEAN NEUTROPHIL VOLUME IN ACUTE INFECTION

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Background. Review of peripheral blood smears can yield important diagnostic information through the identification of the morphologic changes characteristically seen in reactive neutrophils during infection. This approach, however, is labor-intensive and time-consuming because it requires manual examination. Furthermore, the results are subjective because they depend on human interpretation, and only a few hundred cells can be analyzed for any given sample. The Coulter LH 780 hematology analyzer (Beckman Coulter, Fullerton, CA) has the ability to measure specific parameters of neutrophil populations like mean and standard deviation (SD) of cell volume (MVI,SDVI), conductivity (MCI,SDCI), and light scatter (MSI, SDSI). These so-called positional parameters (PP) can detect morphologic changes in neutrophil population and can be an additional indicator for diagnosing acute infection. *Aims.* To investigate the value of the neutrophil MVI generated by VCS technology of the Coulter LH 780 hematology analyzer, as an additional predictor of acute infection. *Methods.* Total white blood cell count, percentage of neutrophils, and positional parameters data from 508 patients with positive blood cultures and from 54 age-matched healthy control subjects were prospectively analyzed. We then studied whether changes in MVI correlated with the type of microorganism. Positive cultures were subdivided to gram (+) or gram (-) microorganism. The PP was obtained by the Coulter LH 750 hematology analyzer (Beckman Coulter, Fullerton, CA). Comparisons between means were performed by analysis of variance. Comparison between 2 means was performed by using the Student t test. A P value less than 0, 05 were considered significant. *Results.* Table. *Summary/Conclusion.* 1) The MVI increases in acute infection, while MSI decreases. 2) Using an MVI cutoff of 150, as in bibliography, seems that MVI is a good predictor of acute infection. 3) The MVI increase correlated significantly both with gram (+) and gram (-) microorganisms but it was greater at gram (-) microorganisms (P=0.16). 4) As a quantitive parameter, the MVI has potential use as an additional indicator for the diagnosis of acute infection.

Table.

	Control	Patients	P	Gram +	Gram -	P
Number	54	508		186	322	
MVI mean	146,17	162.71	<0.001	161.6	163.3	0.16
MCI "	145,85	140.89	<0.001	141.3	140.5	0.1
MSI "	146,06	136.57	<0.001	136.7	136.5	0.851

1935

ENHANCEMENT OF CHEMOTACTIC ACTIVITY IN NEUTROPHILS BY HOT WATER EXTRACT FROM AGARICUS BLAZEI MURILL

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Background. Hot water extract of Agaricus blazei Murill (ABM) is generally known in anti-tumor and immune activities. In previous studies, we reported the increase of number of neutrophils in ABM injected mice. However, the mechanism of increase of neutrophils by ABM is not clearly understood. *Aims.* In this present study, we investigated the mechanism of increased neutrophils, functions of induced neutrophils by ABM and effective comportment of ABM. *Methods.* ABM was provided by Kyowa Wellness Co. Ltd. ABM was freeze dried and adjusted various

concentrations by PBS. ABM was fractionized from fraction number 1 (Fr.1) to 6 by gel filtration using a Shodex OHpak SB-802 HQ column and HPLC. Neutrophils were obtained from guinea pig blood. Neutrophils were isolated from blood of guinea-pig using ficoll-paque. Chemotactic activity for neutrophils was analyzed by EZ-TAXIScan. Productions of reactive oxygen species were measured by FACS using Hydroethidine and 2',7' -Dichlorofluorescin diacetate. Phagocytosis was measured by FACS using FITC labeled Latex beads. *Results.* ABM showed chemotactic activity for neutrophils. Velocities as an indicator of chemotactic activity in migrated neutrophils were 0.31±0.07 μm/sec. (mean±S.D.), 0.28 ±0.07 μm/sec. in ABM at concentration of 50, 100 mg/mL and 0.11 ±0.09μm/sec in control. There were significantly (P<0.001) difference between ABM and control. Radians as an indicator of chemotactic activity in migrated neutrophils were also significantly (P<0.001) increased by ABM at concentration of 50 and 100 mg/mL compared with control. Velocity and radian of migrated neutrophils were significantly (P<0.001) increased by Fr.2, 3 or 4 of ABM compared with control. Fr.3 of ABM showed the strongest chemotactic activity for neutrophils in the both of velocity and radian in migrated neutrophils. Effective component was Fr.3 in ABM and the effective component was substance of approximately from 50 to 200 in molecular weight. Productions of O2- or H2O2 from neutrophils were significantly (P<0.01) increased by ABM compared with control. Phagocytic activity of neutrophils was significantly (P<0.01) enhanced by ABM compared with control. *Conclusion.* Neutrophils were migrated by chemotactic activity of ABM and ABM showed potent activity for functions of neutrophils. The mechanism of increased neutrophils by ABM was due to chemotactic activity of ABM. These results suggest that increase of number and activation in neutrophils by ABM are useful to prevent infection from bacteria.

1936

GAUCHER'S DISEASE TYPE 1, ABOUT AN UNICENTRIC STUDY OF 4 PATIENTS OF LATE ONSET

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Introduction. Gaucher's disease is an uncommon inborn recessive autosomal disease due to deficient activity of the lysosomal enzyme Beta glucocerebrosidase. The not neurological form (type1) is more frequent than the neurological forms (type2 and 3), it is generally diagnosed during the 1st or 2nd decade. We report 4 cases affected by Gaucher 's disease type 1 in whom the discovery was late and made in the adulthood.

Table.

	Case 1	Case 2	Case 3	Case 4
Age (years)/sex	34/ F	35/ F	45 /M	72/ M
Symptoms in the discovery of the disease	Sense of abdominal fullness	Anaemic syndrome	Sense of abdominal fullness	Anaemic syndrome
Consanguinity	No but endogamy	5th degree	No but endogamy	Not precised
Antecedents	-	-	-	Post traumatic Splenectomy
Clinical examination	Splenomegaly 5 cm	Hepatomegaly Splenomegaly	Splenomegaly	Splenectomy
WBC 10^9/l	2980	3200	1800	5800
Hemoglobin g/dl	12.1	9.7	7.8	8.1
Platelet Giga/l	130	119	20	11
Cytology and histology of bone marrow	Gaucher's cell	Gaucher's cell	Gaucher's cell	Gaucher's cell
Other histology with Gaucher's cells	Spleen Liver		Spleen Liver	Liver
Enzymatic dosage	Not done	15 % of normal activity	Not done	Not done
Molecular biology	N370S/N370S	N370S/N370S	N370S/L444P	Not done
Treatment	Watching	Total splenectomy	Partial splenectomy	Transfusion
Evolution/Survival after diagnosis	Alive stable/1year	Alive stable/1years	-Improvement of cytopenias -Bone fracture -Parkinsonism -Still alive /8years	Died by pneumonia /6 months

Patients and Methods. The positive diagnosis of the Gaucher's disease held on the presence of Gaucher's cells by medullary cytology and /or histology (spleen, liver and/or bone marrow) and/or low rate of Béta glucocérébrosidase < 15% of normal activity without neurological disorders. *Results.* See Table joint (image). *Discussion.*The ages of our patients ranges from 34 and 71years, it is indeed a form of late-onset. In our series, the disease is paucisymptomatic: the splenomegaly and/or the signs of medullary insufficiency are the most represented. The replacement treatment is unavailable in our country, that's why we have used splenectomy to improve cytopenias. This latter would have deteriorated and precipitated the arisen of bone's complications at the patient number 3, unlike the patient number 4 the post traumatic splenectomy would have delay the diagnosis. The analysis of the mutations showed that N370S is the most frequent in our series, homozygous in two cases. This mutation allows predicting evolution towards osseous complications and Parkinsonism such is the case of the patient number 3 which is heterozygous for this mutation.

1937

BROTHER & SISTER WITH TWO OTHER CASES OF CHEDIAK HEGASHI SYNDROME WITH DIFFERENT PRESENTATIONS IN MY CHILDREN HOSPITAL - ZAGAZIG UNIVERSITY - EGYPT

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Case-1-Boy. History: Male, A.R.M, 3 ys, 1st kid, from Ismailia (Egypt),Positive consanguinity.Condition started with bloody diarrhea at 1.5 years and during next 1.5 years,he had recurrent throat infection with fever. At 3 years, the patient developed nausea,vomiting followed by jaundice&abdominal enlargement then oliguria. Then pt had severe recurrent abscesses with pain&difficulty during sitting&standing. Examination. Generalized skin darkening (mainly extremities) like-dirt, Change hair color specially forelock (black to gray&White), Two face abscesses (Nose&Cheeks), Horizontal nystagmus since birth, Hepatosplenomegaly and Massive ascitis. *Case-2-Girl(Boy's sister).* History: Female, A.R.M, 10 months, 2nd kid, from Ismailia (Egypt), Positive consanguinity. Condition started at 3 months with constipation&recurrent throat infections&fever responding to antibiotics&antipyretics. Examination. Darkening of the skin (Mainly in face) with change hair color specially forelock(black to Silver-gray&White). HLA Matching Testing: Boy: A(A29),B(B7,B62,BW6),C(CW1,CW6). Girl: A(A2,A34).B (B41,B50,BW6),C(CW2,CW6). Father: A(A29,A34),B(B41,B62,BW6),C (CW1,CW2). Mother: A(A2,A29),B(B7,B50,BW6),C(CW6). *Case-3-Girl.* History: Female, M.A.E., 2 ys, 3rd kid, from Zagazig (Egypt), Positive consanguinity. Condition started at 1 y with recurrent G.E. with bloody diarrhea&vomiting then constipation at 1.5 ys then pneumonia&effusion with abscesses in forearm&neck with history back abscess at 40 days.After that,hair color change&hepatosplenomegaly. *Case-4-Girl.* History: Female, S.O.I., 2 ys, 4th kid, Zagazig (Egypt), Positive consanguinity&older sibling with similar condition died at 10 yrs from severe recurrent infections. At 1y,recurrent throat&G.I. infections.At 1.5 ys,she had severe irritability&refusal feeding, cough, coryza&severe oral moniliasis. Examination: Albinism, red iris, horizontal nystagmus, svere oral moniliasis. Differential diagnosis: Albinism, Bacterial Mouth Infections, Cutaneous T-Cell Lymphoma, Griscelli Syndrome, Pyoderma Gangrenosum, Investigation for all four cases: CBC:cytoplasmic inclusions in lymphocytes, monocytes and neutrophils. B.M.: Hypercellular BM with vacuoles & giant granules in cytoplasm of eosinophils, eosinophilic myelocytes and lymphocytes suggesting CHS. Skin biopsy specimen:normal but may show melanin macroglobules and perhaps sparse dermal melanin. (N.B.:Almost the same findings in all four cases).

1938

CLINICAL ASPECTS AND DIAGNOSIS OF PAROXYSMAL NOCTUTNAL HEMOGLOBINURIA (PNH)

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Background. The PNH is a rare disease, acquired, chronic, is associated with clonal expansion of one or several hematopoietic stem cells carrying somatic mutations acquired PIG-A gene. Two proteins play a role in determining the CD55 DAF and CD59 MIRL. Its clinical presentation is polymorphous just be an array of hemolysis or bone marrow. Recent studies have shown that 20-25% of aplastic anemia have a PNH clone. We report 35 observations or PNH clone was searched by flow cytometry. *Aims.* Flow cytometry is a new technique used recently in

our laboratory for the positive diagnosis of PNH. *Methods.* Clinical study, biological, cytological test Dacia 03cas Ham, sucrose 01 test cases and immunophenotyping by flow cytometry carried out on a BD FACS Calibur cytometer 04 colors. This technique assesses the degree deficiency of CD 55 and CD 59 on neutrophils and red blood cells. The definitive diagnosis in practice is the detection of more than 5% of cells deficient in CD55 and CD59. *Study materials.* These 17 women and 18 men with an average age of 33 years (16-62 years). The reasons for consultation: anemia: 18 cases, bleeding: 08cas, low blood: 03 cases, pregnancies: 02 cases, bone pain: 02 cases and cough: 01 case and fortuitous: 01 cases. A bone puncture biopsy done in 26 cases: Aplastic anemia: 17 cases, hypoplasia: 07cases, normal: 02 cases, MDS: 01cas. *Results.* The deficit on the 02 proteins CD55 and CD59 in both cell populations was found in 12 cases or 34% of cases typed. This deficit was noted in 04 women and 08 men with an average age of 33 years (17-49 years). Clinics: anemia: 07 cases associated with episodes of jaundice: 02 cases and dark urine: 01 case, severe anemia in pregnancy: 01 case, bleeding: 02 cases associated with lumbar pain and signs of renal blood 01 case. BOM: Pancytopenia: 10 case and bicytopénie: 01 case. : 08 cases Aplastic anemia: 04 cases, bone marrow hypoplasia: 02 cases, ordinary: 02 cases and myelodysplasia: 01 case. The Dacia Ham test was performed in 03 cases: positive and a test Sucrose has been conducted in one case: positive. Indicative Table. *Commentary.* In our study, the CMF has been instrumental in the diagnosis of PNH. It should be performed before any bone marrow, an array of hemolysis in Coombs negative and unexplained recurrent thrombosis in search of a PNH clones because it is more sensitive and specific than traditional tests.

Table. Results.

	Case	1	2	3	4	5	6	7	8	9	10	11	12
GB %	CD 59	53	10	17	15	22	12	13	49	07	44	63	34
	CD 55	84	96	94	96	67	14	29	49	09	85	69	12
GR %	CD 59	15	59	49	65	27	12	43	09	07	08	24	57
	CD 55	27	68	56	82	38	14	67	08	15	16	24	41

1939

PEDIATRIC ACUTE IDIOPATHIC THROMBOCYTOPENIC PURPURA: HOW LONG DO WE HAVE TO WAIT FOR A NEW AUTOIMMUNE MANIFESTATION?

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Background. As well known in literature Chronic immune thrombocytopenic purpura (ITP) is often associated with other autoimmune diseases. Otherwise, often reported only as sporadic experience is the relationship between acute ITP and other autoimmune manifestations. *Aims.* The aim of our study is to evaluate the incidence of other autoimmune conditions in a cohort of paediatric patients with chronic or acute ITP. *Methods.* Since December 2007 till December 2009 we observed 16 children (8 M and 8 F) mean age 6yrs, ranged between 8 mts-15 yrs, 8 with chronic ITP and 8 with acute ITP (at least 2 yrs of follow up). All patients were periodically screened for coeliac disease (CD) determining the anti transglutaminase antibodies (TTG) and anti endomisial antibodies (EMA), and for other autoimmune conditions determining serum levels of antinuclear antibodies (ANA), anti smooth muscle antibodies (ASMA), anti mitochondrial antibodies (AMA), C3 and C4, thyroidal function, anti tireoperooxhidase antibodies (AbTPO) and anti tireoglobulin antibodies (AbTg), Direct Coombs test, and anti gastric parietal antibodies (APCA), and anti DNA antibodies. *Results.* in the group of 8 patients with chronic ITP 2/8 has shown thyroiditis, 2/8 CD, 1/8 psoriasis, 1/8 no clinical manifestations but was serologically positive for Direct Coombs Test and APCA; 2/8 had no clinical or serological manifestations of autoimmune disease. On the other hand in the group of Acute ITP 2/8 has shown positive Direct Coombs test with no clinical manifestations and/or signs of haemolysis; 1/8 was serologically positive to Anti-DNA completely asymptomatic; 5/8 serologically and clinically negative. All the 3 patients developed the autoimmune condition during the follow up, after more than 1 year from complete remission (CR). (See Table below). *Summary and conclusions.* Our study seems to confirm the high incidence of second or more autoimmune conditions in Chronic ITP. Otherwise it seems to show an high predisposal condition to autoimmunity also in CR patients with Acute ITP. It should be necessary to extend for more than the usually indicated 2 years the survey for autoimmunity in Acute ITP. Further studies, possibly multicentric, will be necessary to confirm our data and evaluations.

Table.

ACUTE ITP	CHRONIC ITP
• 2 / 8 POSITIVE COOMBS TEST •1/8 POSITIVE ANTI DNA	•2/8 THYROIDITIS •1/8 PSORYASIS •1/8 NO CLINICAL MANIFESTATION BUT POSITIVE COOMBS TEST AND APCA •2/8 NO AUTOIMMUNE MANIFESTATION

1940

DO WE KNOW AN IDEAL PATIENT WITH IMMUNE THROMBOCYTOPENIA FOR RITUXIMAB TREATMENT?

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Introduction. Immune thrombocytopenia (ITP) is an autoimmune disease characterized by low count of platelets and determination to bleeding. Standard initial treatment options for ITP are corticosteroids and intravenous immunoglobulins. Adult patients with chronic disease are additionally treated with immunosupresive agents (ciclosporin, mycophenolate mofetil, azathioprin, and other). Rituximab is chimeric humanized monoclonal antibody against $CD20^+$ B lymfocytes. It is currently indicated for the treatment of malignant lymphoma, but because the rituximab targets are B lymphocytes it is also used for treatment of autoimmune diseases such as systemic lupus erytematodes and rheumatoid arthritis. In hematology, rituximab is also more often used for the treatment of autoimmune hemolytic anemia, thrombotic thrombocytopenic purpura and immune thrombocytopenia. There are many case reports published about the treatment of ITP with rituximab, however we do not know yet exactly what sort of patients is ideal to receive this therapy. *Methods.* We retrospectively analyzed data of 36 patients with diagnosis of ITP who were treated with rituximab (375 mg/m^2 weekly, four-times; standard infusion-related side-effects prophylaxis) in our center between years 2000 to 2009. In this analysis we were looking for epidemiological data such as age, sex, time from ITP diagnosis to rituximab, and number and a kind of concomitant medication. We particularly analyzed the effect of rituximab with regards to the length of duration of therapeutic response. *Results.* All our patients completed treatment. Therapeutic responses (analyzed month 2 after rituximab treatment) were as follows: complete remission (CR), n=10 (28%); partial remission (PR), n=13 (36%); and no response (NR), n=13 (36%). There was no significant difference in response rate in splenectomized and non-splenectomized patients, however a trend to better effect of rituximab was observed in patients without splenectomy. Patients with splenectomy (n=17): CR, 4 (24 %); PR, 6 (35 %); NR, 7 (41 %). Patients without splenectomy (n=19): CR, 6 (31.5%); PR, 7 (37 %); NR, 6 (31.5%). Also, there was no significant difference in response rate in patients divided according to the time from diagnosis to the rituximab administration (2 years and less *vs.* more than 2 years). In patients who reached PR or CR within 2 months after rituximab administration (n=20), the median progression-free survival was not reached. Only one of these patients patient progressed. *Summary.* Rituximab usage for adult ITP patients is one of well-tolerated 2nd line therapy options. We observed that patients with good initial response (CR, PR) tend to achieve a durable response. On the other hand, in our analysis splenectomy, a number of previous treatments, and time from diagnosis to rituximab administration were not significant predictors of favorable response. Additional studies are needed to determine an optimal timing of rituximab usage.

1941

LONG-TERM FOLLOW-UP ANALYSIS OF HELICOBACTER PYLORI ERADICATION TREATMENT FOR JAPANESE IDIOPATHIC THROMBOCYTOPENIC PURPURA PATIENTS

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Background. Although many reports revealed that Helicobacter pylori (HP) eradication is effective for Japanese idiopathic/immunological thrombocytopenic purpura (ITP) patients, long-term outcome of the HP eradication treatment have been reported very little. *Aims.* We aim to clarify the effectiveness of HP eradication treatment for Japanese HP-ITP in terms of long-term outcome. *Methods.* One-hundred nine patients who showed under 100×10^9/microliter platelets at diagnosis and who could be observed for more than 6 months among 129 patients diagnosed as ITP from July 1998 to December 2007 in our hospital were analyzed. Watchful wait (w/w); 35, HP eradication; 36 (*de novo*; 22 and relapsed/refractory; 14) and other treatment (steroid hormone or splenectomy); 38 patients were included. We analyzed; (1) Complete remission ratio (CRR) and CR duration of each groups, (2) relapse rate and progression-free survival of HP eradication, and (3) clinical outcome of HP eradication in each *de novo* HP-ITP and relapsed/refractory HP-ITP. This study was performed under the guidelines for epidemiologic study established by the ministry of health, labor and welfare in Japan. *Results.* (1) CRR of HP eradication is superior to other treatment, that is 47.2%/55.2% at 2 months and 66.7%/60.5% at 6 months (P=0.0438), respectively. Mean CR duration is 1264 (range; 208-1846) days in HP eradication and 1050 (210-3337) days in other treatment. (2) No relapsed patient was observed in HP eradication and mean PFS is 1303 (208-2020) days. (3) In HP eradication treatment, CRR at 6 months is 59.1% in *de novo* HP-ITP and 78.6%% in relapsed/refractory cases (P=0.0093). *Sumarry/Conclusions.* HP eradication treatment for Japanese HP-ITP patients showed comparably high CR ratio with very high progression-free survival. We also have found out that about 10% of responders show slow response to HP eradication who could not obtain CR at 2 months but obtain CR at 6 months. It might be possible to assume that reduction of stimulation of HP antigen to immune system occur in indirect and slow manner.

1942

WITHDRAWN BY AUTHOR

1943

TNF-α, TGF-β1, IL-10, IL-6 AND IFN-γ GENE POLYMORPHISMS IN PATIENTS WITH IDIOPATHIC THROMBOCYTOPENIC PURPURA

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Background. The polarization of Th1/Th2 towards Th1 contributes to the ethiopathogenesis of idiopathic thrombocytopenic purpura (ITP). Cytokine polymorphisms may play crucial roles in the pathogenesis of ITP. *Aims.* The purpose of this study was to investigate whether tumor necrosis factor (TNF)-α (-308), transforming growth factor beta 1 (TGF-β1) (codons 10 and 25), interleukin 10 (IL-10) (-1082, -819, and -592), IL-6 (-174) and interferon gamma (IFN-γ)(+874) polymorphisms may be responsible in part for genetic susceptibility and treatment of ITP. *Methods.* Genotyping was performed in 71 adult patients with chronic ITP and in 71 healthy individuals by PCR-SSP method. *Results.* Of these polymorphisms, frequency of the TNF-α AG genotype and IFN-λ. AA genotypes were significantly higher in ITP patients than in healthy controls (P=0.016). Frequency of the TGF-β1 (codon 10) TT genotype was significantly lower in ITP patients than in healthy controls (P=0.016). TNF-α AG genotype was significantly higher in steroid-refractory and splenectomized cases relative to steroid-responsive (complete response and remission) ones at the end of one year. When we compared the steroid responsive cases that were at the first steroid course with the 12 cases who were in remission, but in whom no complete response was achieved, we found their frequencies to be 12 (20.3%) and 6 (50%) in IFN-γ AA genotype, respectively. *Conclusions.* With these findings, TNF-α(-308) AG, TGF-β1(codon 10) TT, IFN-γ(+874) TT genotypes were detected to be the genes with tendency to ITP. It is shown that genotypes of TNF-α(-308) AG and IFN-γ(+874) AA might be important in steroid treatment for ITP in Turkish patients.

1944

RESPONSE TO SPLENECTOMY FOR ADULT PATIENTS WITH PRIMARY IMMUNE THROMBOCYTOPENIA FAILING TO FIRST- AND SECOND-LINE THERAPIES: LONG-TERM FOLLOW UP

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Background. Primary immune thrombocytopenia (ITP), also known as idiopathic thrombocytopenic purpura, is an immune-mediated acquired disease of adults and children characterized by transient or persistent decrease of the platelet (Plt) count and, depending upon the degree of thrombocytopenia, increased risk of bleeding. The major goal for treatment of ITP is to provide a safe platelet count (e.g. one that prevents major bleeding) rather than correcting the platelet count to normal levels. For patients with ITP failing to first- and second-line treatment splenectomy is the best therapy option. *Patients and methods.* between 1991 and 2009. 15 patients (pts) (sex: 10 men; 5 women; median age 30 years) with ITP failing to first- and second-line therapy were splenectomized. The median time from diagnosis to splenectomy was 6 months (range 3 to 14). All patients were treated before surgery without results (steroids, androgens, vincristine, cyclosporine, IVIG, Rituximab). Only in 4 (40%) out of 10 patients treated before splenectomy with high-dose immunoglobulins, increased platelet count over 100×10^9/L have observed. *Results.* immediately following splenectomy in 13 patients (86.67%) increased platelets count happens. We have noticed postoperative complications in 3 patients (20%) - in 2 of them (13,3%) appearance of haemathoma in spleen loge with consecutive infection (subphrenical abscessus), while in 1 patient (6,7%) we have observed pancreatic fistula. Those particular complications have been solved successfully (fistula after 8 months). Decreasing platelet number was objected in second postoperative week in 1 patient with partial response, those one with subphrenical abscessus, with renewal appearance of hemorrhagic syndrome and we have continued with application of IVIG in combination with steroids, androgens and (following healing of abscessus) even with chemotherapy according to COP regimen (2 cycles). Six months after splenectomy, partial remission has occurred (4 years after splenectomy patient spontaneously reached complete remission). Other patient is still in the stabile partial remission after the splenectomy (Plt above 70 and below 100×10^9/L). After the long-term follow up (2 to 19 years, median time 7,6 yrs) there were neither any complication nor relapses of the ITP. *Conclusion.* This study indicates that more than 86.6% of patients with ITP failing to first- and second-line therapy could achieved long term remission with minimal risk after the splenectomy.

1945

POTENTIAL APPLICATIONS OF ROMIPLOSTIM IN THE DAILY PRACTICE

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Background. Romiplostim is a MPL ligand recently approved as treatment for refractory immune thrombocytopenia (ITP) and currently explored in patients with low platelet counts from other causes. *Patients and methods.* We analyzed 17 consecutive patients who received Romiplostim in our center. Twelve patients were treated for refractory ITP, 4 for myelodysplastic syndrome (MDS) and 1 for severe thrombocytopenia after allogeneic stem cell transplantation (Allo-SCT). The latter 5 patients received Romiplostim on a compassionate basis. The drug was administered subcutaneously at an initial dose of 1 mcg/kg (except for two MDS patients who started with a dose of 300 mcg). The dose was increased by 1 mcg/kg weekly until achieving a platelet count above 50×10^9/L. Platelet response was defined as a platelet count of $>50\times10^9$/L and/or doubling of the pre-treatment count, in the absence of any rescue medication within the last 8 weeks. *Results.* In the 12 patients with ITP (median age 70 years, range 37-81), the median number of therapy lines before Romiplostim was 2 (1-4). Median platelet count at starting Romiplstim was 17×10^9/L (range 5-180). Ten patients achieve a complete response (83%) after a median of 2 weeks (range 1-4). The median platelet count at last follow up was 103×10^9/L (19-550). All except 2 patients continue under Romiplostim after median treatment duration of 13 weeks (range 3-34). In 1 patient the treatment was discontinued because of thrombocytosis and in another was stopped due to lack of response. Of the 4 MDS patients (median age 78 years, range 70-87), all had previously received 5-azacytidine. Median platelet

count before Romiplostim was 7×10^9/L (range 4-16), with all patients being transfusion dependent. One patient is in complete remission achieved with a dose of 6 mcg/kg and the platelet count is 84×10^9/L 24 weeks after starting treatment; another patient failed to respond and Romiplostim was discontinued after increasing the dose up to 7 ug/kg during 10 weeks. The remaining two patients are under treatment since they have only received 4 doses of the drug; of note, in one of them the platelet count has already increased from 4 to 39×10^9/L platelets and in the other from 7 to 20×10^9/L platelets with the two of them free of transfusions. The patient who received Romiplostim as therapy for persistent thrombocytopenia after Allo-SCT did not improve and the agent was discontinued after 4 weeks. Overall, there were 3 patients who showed a transient thrombocytosis. Another occasionally observed side effect was mild injection site reaction. *Conclusion.* Romiplostim has different potential indications. The use of this agent following the current recommendations appears safe and may be useful in different conditions leading to thrombocytopenia.

1946

THROMBOCYTOPENIA IN PATIENTS HOSPITALIZED IN INTENSIVE CARE UNIT (ICU)

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Background. Alterations of hemostasis and blood counts are common in patients admitted to ICU. Thrombocytopenia increases the morbidity and mortality being its aetiology very varied. *Aims.* 1) analyze the incidence of thrombocytopenia in patients admitted to ICU: clinical, laboratory and therapeutic factors. 2) Classification of patients undergoing cardiovascular surgery following the trend in platelet count. *Methods and patients.* A retrospective study by reviewing medical records of patients with thrombocytopenia (platelets $<125\times10^9$/L) admitted to four ICUs of our hospital during a 40 days period, classified as mild (100-50×10^9/L), moderate (50-20×10^9/L) and severe ($<20\times10^9$/L). Evaluation of patients undergoing cardiovascular surgery with cardiopulmonary bypass, classifying them according to changes in the platelet count until day 10 post surgery: type 1 pattern (biphasic evolution) and type 2 pattern (persistent decline), and their involvement with the development of Induced Thrombocytopenia Heparin (HIT) as the patterns presented by Selleng *et al.* (J Thromb Haemost 2009; 8: 27-29). *Results.* 237 patients admitted during this period, 50 showed thrombocytopenia (21%). Cumulative incidence: 19.8% in 40 days, excluding those who previously had his income. Men 56%, Women 44%, mean age 70.7 (extremes 19-88). Depending on different UCI: Coronary 9 patients, mean age 67.5, cardiovascular 25 patients, mean age 70.0; Polyvalent 11 patients, mean age: 65.8; Traumatology 6 patients, the mean age: 75.6. Reason for admission: 32% valve replacement, infection 14%, ischemic heart disease 14%, respiratory 8% hepatectomy 8%, coronary artery bypass surgery 8%, ruptured aneurysm 4%, bleeding 4%, decreased consciousness 4%, other 6 %. Mild thrombocytopenia: 94%, moderate: 0%, severe: 6%. Nadir figure rating: 90x10 9 / L. Average time duration of thrombocytopenia: 4.2 days, median: 2.5 days. Etiology surgery with cardiopulmonary bypass 42%, infectious 24%, liver pathology 14%, drug toxic-10%, blood disorder 6%, other 6%. Distribution due to drug: abciximab 8%, tirofiban 2%. Incidence bleeding: 30%, with major bleeding (10%) located in: mediastinal 2%, hematoma in back with 2% drop in hematocrit, HDA 2%, brain 2% pulmonary and 2%, the rest were minor bleeds. Thrombotic phenomenon not observed. Transfusion: CH 54%, CP 22%. Incidence of coagulopathy: 30%. Regarding the administered treatment: plasma 12%, vit K 8% FVIIr and other measures 2%, Vitamin K and plasma 2%. The attributable mortality: 10 patients (20%). Of 78 patients undergoing cardiac surgery showed thrombocytopenia 25 (32%) and all of them showed a type 2 pattern of platelet count, and in no case the etiology was HIT. *Conclusions.* 1) Surgery bypass is the most common cause of thrombocytopenia, because of our center nature, followed by infectious causes. 2) In most cases, thrombocytopenia is mild and transient, short-lived, and have not needed treatment. 3) The classification of patients according to the proposed standards could have a negative predictive value in the case of type 2 pattern, though larger series are needed for a better evaluation. 4) In the critically ill patient with multiple pathologies, thrombocytopenia is a factor with impact on morbidity and mortality.

1947

ASSOCIATION OF PLATELET PARAMETERS AND SERUM COMPLEMENT WITH COAGULASE-NEGATIVE STAPHYLOCOCCAL BACTEREMIA: A CROSS-SECTIONAL STUDY

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Background/Aims. Bacteremia and systemic inflammatory response syndrome involve inflammation, thrombopoiesis and haemostatic mechanisms. Mean platelet volume (MPV), platelet distribution width (PDW), platelet count and their correlations with serum complement levels (C3 and C4) and C-reactive protein (CRP) have not been studied in depth. The aim of this study was to explore the correlation of platelet indices with serum complement levels and CRP, and to determine their significance in the evolution of coagulase-negative staphylococcal (CNS) bacteremia. *Methods.* We have evaluated 18 incident cases of coagulase-negative staphylococcal bacteremia (10 men and 8 women, 27-80 years) and an equal number of hospital controls with minor non-infectious and non-neoplastic conditions, individually matched for age (±5 years), gender and time at diagnosis. To assess thrombopoiesis and inflammation, we have determined platelet indices and leucocyte count using Sysmex 9000 analyser. C3, C4 and CRP levels were determined using immunonephelometry. Statistical analysis of data was performed with SPSS® version 17 for Windows software. *Results.* Significantly low mean values of serum C3 complement levels ($P<0.001$) along with significantly high mean values of platelet count, MPV, PDW, platelet-to-large-cell ratio, leucocyte count and CRP were shown in patients with CNS bacteremia in comparison to their controls ($P<0.05$). Serum C4 didn't present any significant difference ($P=0.91$). There was a significant negative relationship between CRP and serum C4 in patients with bacteremia ($r=-0.49$, $P<0.03$). Adjusting for age, gender and CRP levels, the presence of CNS bacteremia is a statistically significant predictor of MPV levels ($P=0.035$). Patients with CNS bacteremia presented normal platelet indices as well as serum C3 complement levels after resolution of the infection. *Summary/Conclusions.* Platelet indices and especially MPV may provide an accessible and adjunct clinical useful index to the current laboratory parameters for estimating the degree of inflammation and the degree of bone marrow function in patients with CNS bacteremia, predicting also resolution of the infection.

1948

TIME TO SPLENECTOMY FAILURE IN PATIENT WITH REFRACTORY CHRONIC IMMUNE THROMBOCYTOPENIC PURPURA

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Introduction. Splenectomy has been perceived as a potentially curative treatment for chronic immune thrombocytopenic purpura (ITP). Schwartz (2003) demonstrated that the risk of relapse is between 33%-55% at patients with longterm follow-up. Splectomised patients are at risk for early and late surgical complications, infections secondary to impaired immunity, thrombotic or cardiovascular disease, pulmonar hypertension. Splenectomy requires preoperative vaccinations , general anesthesia, antibiotic prophylaxis and subsequent longterm vigilence with antibiotic treatment. *Obiective.* To decribe the time from splenectomy to splenectomy failure among patients with chronic ITP enrolled in the clinical program(prior treatment with dexamethazone -high dose). *Methods.* Date of splenectomy and prior medications for ITP were reviewed in patients enrolled in 3 clinical trials using dexamethazone highdose. Splenectomy was considered to have failed upon administration of the first treatment for ITP after surgery or than patients were not able to taper or interrupt concomitent ITP treatments in the 15 days following splenectomy. Of the 89 patients enrolled in the ITP cohorte, 32 (27%) were splenectomised and 44 were evaluable for this analysis. The analysis does not describe overall effectiveness of splenectomy as the patients were limited to splenectomy failure that required additional treatment for ITP. *Results.* 21% of patients required ITP medication within one year from the splenectomy. Five years after the splenectomy, 24% of the patients still had a response. This percentage decreased to 13% after 10 years. *Conclusions.* this retrospective analysis demonstrates that the success of splenectomy diminishes over time at patients requiring further ITP treatment, most splectomised patients who relapsed in 5 years. The treatment of chronic ITP has advanced as more

data of the safety and efficacy of new medications (Rituximab or Eltrombopag).

1949

COMPARISON OF PLATELET ACTIVITY IN PATIENTS WITH DIABETES MELLITUS AND SUBJECTS WITH PRE-DIABETES

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Background. Large platelets are more active and hence more thrombogenic than smaller ones. Mean platelet volume (MPV) is an indicator of platelet thrombogenic function. It has been reported that patients with diabetes mellitus have significantly higher MPV values which is probably associated with increased cardiovascular morbidity. Values of fasting glucose ranging from 100 mg/dL to 125 mg/dL are considered, according to the diagnostic and classification criteria issued by the American Diabetes Association (2008), as a pre-diabetes state and a risk factor for future diabetes. The MPV in patients with impaired fasting glucose (pre-diabetes) has been lately under investigation. *Aims.* The objective of this study was to evaluate MPV in subjects with pre-diabetes compared with diabetic patients and normoglucemic control subjects, and to investigate the potential associations between MPV, fasting blood glucose (FBG) and glycated hemoglobin (HbA1c) measurements. *Methods.* A total of 122 patients from the Outpatients Department of AHEPA University Hospital were enrolled to the study. They were divided into three groups. Group 1 comprised of 50 (15 males and 35 females) diabetic patients (FBG>126 mg/dL, HbA1c>6%); group 2 of 48 (20 males and 28 females) pre-diabetic patients (FBG =100-125mg/dL, HbA1c<6%); group 3 of 24 (13 males and 11 females) normoglucemic healthy subjects. Exclusion criteria for entry into the study was the presence of co-existing thrombotic or hematological disease and the use of anticoagulant agents. MPV and platelet count were measured with hematological analyser HE-2100 SYSMEX, ROCHE, glucose levels with phasmatophotometric method with Modular P 800, ROCHE and glycated hemoglobin with high performance liquid chromatography-HPLC with the semiautomatic system VARIAN, BIORAD. Statistical analysis was made by unpaired Student's t test and correlations were tested using Pearson correlation analysis. *Results.* The laboratory parameters are reported in Table 1. MPV was significantly higher in the diabetic and the pre-diabetic groups than in the control group (P<0,001). The analysis showed that there was no significant difference between the platelet count of the three groups. MPV was positively but not significantly correlated with fasting blood glucose and HbA1c in diabetic and pre-diabetic groups. *Summary/Conclusions.* The study shows a stepwise increase in MPV, which is a marker of platelet thrombogenic function and activity, from a non-diabetic to a pre-diabetic and then further to a diabetic population. In the same samples of blood no significant change in platelet count was found. No significant correlation between MPV and glycemic control (fasting blood glucose and HbA1c) was observed. As the increase in MPV is considered an independent risk factor for myocardial infarction, patients with pre-diabetes may be at an increased risk of cardiovascular event as well as the diabetic ones.

Table 1. Platelet activity and diabetes parameters in diabetes mellitus, pre-diabetic and control group.

Laboratory parameters	Diabetic patients	Pre-diabetic subjects	Control group
Fasting blood glucose (mg/dl)	195,04 ± 67,92	116,17 ± 3,89	93,29 ± 8,14
Glycated hemoglobin (HbA1c, %)	8,08 ± 1,56	5,30 ± 0,41	4,68 ± 0,49
Platelet count (PLT, K/μL)	259,08 ± 68,23	234,39 ± 54,05	230,95 ± 69,62
Mean platelet volume (MPV, fL)	11,28 ± 0,91	11,16 ± 0,95	8,79 ± 0,79

1950

ROMIPLOSTIM FOR THE TREATMENT OF IMMUNE THROMBOCYTOPENIA ASSOCIATED TO CHRONIC LYMPHOCYTIC LEUKEMIA AND MAINTENANCE OF THE RESPONSE WITH TWICE-WEEKLY ADMINISTRATION

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Background. Romiplostim is an Fc-peptide fusion protein. It works intracellularly in a manner similar to that of the naturally occurring thrombopoietin (TPO) to activate the transcriptional pathways, leading to increased platelet production via stimulation of the c-Mpl receptor. Its use is approved for adult chronic immune thrombocytopenia purpura (ITP) splenectomised patients who are refractory to other treatments and as second line treatment for adult non-splenectomised patients where surgery is contraindicated. Approximately 2% of chronic lymphocytic leukemia (CLL) patients develop clinically significant ITP. The diagnosis is made on the basis of an unexplained fall in platelet count in the absence of bone marrow failure due to leukemic infiltration or hypersplenism. We use a second generation non-immunogenic TPO receptor agonist in a case of refractory ITP-CLL. *Case summary.* A 73-year-old man was diagnosed with CLL and immune thrombocytopenia associated in May, 2008. He presented extreme thrombocytopenia with moderate hemorrhagic manifestations that have needed treatment with steroids and immunoglobulin with partial response and rituximab for maintenance of the platelet response. In May 2009, he was admitted to the hospital with severe thrombocytopenia of 3×10^9/L and mucocutaneous bleeding. Treatment with corticosteroids, intravenous immunoglobulin, rituximab and vincristine was started on but thrombocytopenia showed refractoriness. The patient presented a bilateral pneumonia for Stafilococo aureus and a life-threatening digestive hemorrhage. Baseline platelet counts were 5×10^9/L so we decided to begin the treatment with Romiplostim. The initial dose of romiplostim was 3 microg / kg / week/ subcutaneus. Platelet response was observed on day 7 (58×10^9/L platelets) with a cessation of the digestive hemorrhage. Platelet counts increased with once weekly dosing. Patient was able to discontinue the concomitant medications. Durable platelet response (Platelet count ≥ 50×10^9/L during 6 or more of the last 8 weeks of treatment) was maintained with continued romiplostim twice-weekly. Romiplostim was well tolerated. Adverse events were thrombocytosis> 400×10^9/L and worsened thrombocytopenia following romiplostim discontinuation that improved with the restart of the administration. In January 2010, 31 weeks after start of treatment, the platelet counts were 139×10^9/L with Romiplostim twice-weekly without morphologic changes in the peripheral blood smear. We have proposed to the patient the accomplishment of splenectomy. *Conclusions.* Romiplostim has increased and supported the long-term platelet counts in this difficult patient with a good profile of safety. Romiplostim may provide a new therapeutic option for patients with ITP associated to CLL. Clinical trials are required to confirm its efficacy in this indication and the interval of administration.

1951

PLATELET COUNT AS PROGNOSTIC VALUE OF MORTALITY AND ORGANIC DYSFUNCTION IN PATIENTS WITH SEPSIS

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To study the incidence of thrombocytopenia in patients in the emergency department with a diagnosis of severe sepsis and septic shock Correlate the value of platelet count with prognosis and mortality of these patients. Analyze the hematologic organ dysfunction related to that thrombocytopenia in the context of sepsis. *Materials and methods. Study Design.* Retrospective Cohort Sample: We analyzed all adult patients with criteria for the diagnosis of severe sepsis and septic shock in the period from November 2008 to February 2010 in the Emergency Department and Critical Care Hospital Juan Ramón Jiménez de Huelva. All these patients had to meet at the time of the study include the criteria for the diagnosis of hospital plan of management of severe sepsis and septic shock force at the Hospital. In each patient were extracted 2.5 ml of venous puncture, which was placed in tubes used as EDTA. Thrombocytopenia was defined as a platelet count less than 150000 / mm 3 during admission, at 24, 48 and 72 hours. Variables: gender, age, admission and discharge from service, patient type, time of placement in which it met the criteria of severe sepsis, platelet count, destination (high or death) and other parameters as the Score of APACHE (Acute Physiology and Chronic Health Evaluation) and SOFA score (Sequen-

tial Organ Failure Assessment). Statistical Analysis: Data were analyzed by SPSS 16.0. By t-student for independent samples and paired, χ^2, with confidence limits of 95%. *Results.* We analyzed a series of 64 patients, 34 (47%) male and 30 (53%) female, average age of patients was 64±21 years; 31 (59.6%) patients of medical management and 21 (40.4%) of surgical management.The median hospital stay for patients with severe sepsis and septic shock from 8.6±10 days. In these patients, the average platelet count was 96.000/mm 3, being the lowest count 11.000/mm 3. It is noted that mortality increases in patients with thrombocytopenia were statistically significant on admission (P=0.043) at 48 hours (P=0.032) and at 72 hours (P=0.04) at 24 hours was not found statistical significance (P=0.11). The APACHE II score of these patients was 23±7. A low platelet count has also been associated with poor prognosis and increased severity of illness reflected in the APACHE II Score was higher in those critically ill patients with thrombocytopenia than in those not suffering, results were not statistically significant probably by the small sample size. Regarding the distribution of dysfunction and its correlation with haematological dysfunction, was that in both groups the incidence of dysfunction was similar, a fact reflected in the average SOFA Score. *Conclusions.* Thus a decrease in platelet count may indicate poor outcome in critically ill, and that this simple parameter can be trusted to monitor the evolution of patients with sepsis, as several studies suggest that its correction is an important factor for a better prognosis and evolution observed in this study that patients with higher values of APACHE II at 72 hours after admission presented a decrease in platelet count.

1952

DISTURBANCES OF PLATELETS AGGREGATION IN PATIENTS WITH MULTIPLE MYELOMA

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Backgraund. Some patients with multiple myeloma (MM) have haemostatic disorders at presentation, and arising mechanism is not completely explained. It is more frequently belived to be in connection with paraprotein. *Aim.* The aim of this study was proved by investigation with platelets aggregation tests, the incidence of disturbances of platelets aggregation at patients with MM, and to estimated the influence of paraprotein on platelets aggregation at healthy donors, *in vitro*. *Methods.* In this study where included 40 patients with MM. Initially investigation of platelets aggregation induced with ADP, collagen (COL), ristocetin (RIS) and epinephrine (EPI) where done. Paraprotein has been separated by Rivanol method from serum of 9 patients, who had decreased platelets aggregation on used inducers: ADP, COL, RIS and EPI, at presentation. Platelets aggregation in platelet rich plasma (PRP) was measured at 9 healthy donors before and after addition of paraprotein isolated from the patients with MM, induced by same inducers. The indentical test was repeted with addition of human immunoglobulins for intravenous used in PRP at 9 healthy donors. We measured latent time in seconds and maximal platelet aggregation in percent for all inducers. *Results.* Platelets aggregation was disturbed at one third patients. Platelets aggregation was normalized together with disappearing of paraprotein during a treatment. When patients attained remission, their platelets aggregation was normalized. Paraprotein isolated from serum of these patients inhibited platelets aggregation of healthy donors. Average of maximal levels of platelet aggregation has been significantly decreased in PRP of healthy donors after addition of paraprotein when used inducers: ADP (P=0.005), COL (P=0.007), RIS (P=0.004), and EPI (P=0.005). Average of latent time of platelet aggregation was significantly prolonged in healthy donors after addition of paraprotein with inducers: COL (P=0.018), RIS (P=0.033) and EPI (P=0.042). Average of latent time of platelet aggregation was not significantly prolonged after induction by ADP (P=0.169). In comparison, when human immunoglobulins added in PRP of healthy donors, maximal platelet aggregation and latent time were not significantly changed. *Conclusions.* These invastigations have proved that paraprotein leads to haemostatics disorder at patients with MM. Paraprotein isolated from patients with MM, who had decrease platelet aggregation at presentation, significantly decreased platelet aggregation when was added in PRP of healthy donors, *in vitro*. This is confirmed with addition of human immunoglobulins in PRP of haelthy donors, thus, platelets aggregation was not significantly changed.

1953

TREATMENT RESULTS IN PATIENTS DIAGNOSED WITH CHRONIC IMMUNE THROMBOCYTOPENIC PURPURA

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Background. Immune Thrombocytopenic Purpura (ITP) in adults is often a chronic disorder that requires continuous monitoring of platelet counts, bleeding events, and treatment - related side effects. Current ITP treatment options for chronic ITP include corticosteroids, intravenous immunoglobulins (IVIg), rituximab, and clinical supervision in order to prevent bleeding events by appropriate treatments. Splenectomy is reserved for those patients who do not respond or relapse after these medical therapies, or require intolerable doses to achieve safe platelet counts. *Aims.* The purpose of this study is to demonstrate that the result of treatment in patients with chronic ITP does not significantly improve under current standard of care. *Methods.* We present the initial results from a prospective, observational study in patients' previously diagnosed with chronic ITP and consecutively enrolled in this study. Data from patients' charts provided date of 1st ITP diagnosis, treatments received, and medical history. ITP treatments, dose, response, and duration of response were collected prospectively for 12 months. *Results.* The first 150 patients enroled in the study were 46% male; mean age 52 years. During the study evaluation period (at baseline or beyound) 42 patients (28%) were splenectomized and 108 (72%) received ITP - related treatments including corticosteroids (n=79), IVIg (n=25), and Anti-D Antibody (n=4). Our study indicates that the overall response was 68% with 48% CR and 20% PR. The other 32% of the patients were considered in non-remission and 20% of them presented late remission after a median evolution period of approximately 1,3 years. During the evolution, 53% of the patients have relapsed, 41% of the patients have been splenectomized, 26% of the patients which have been splenectomized presented CR and 11% of patients presented PR. The long-term follow-up in CR and PR proves a good, stabil and durable response in time for more than 7 years. The natural coure of the disease is variable. *Conclusions.*Patients with ITP chronic can have a good initial response at the steroid therapy and/or IVIg. Those that fail during this therapy can get a safe and durable response after the splenectomy. Modern treatments like monoclonal antibodies can bring a bigger benefit. Novel ITP intervention are desirable to improve both treatment satisfaction and reported effectiveness in patients with chronic ITP.

1954

EIGHT-YEAR FOLLOW-UP OF PATIENTS WITH IMMUNE THROMBOCYTOPENIC PURPURA RELATED TO H. PYLORI INFECTION

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Background. Thrombocytopenia related to H. pylori infection is a definitive subset of chronic immune thrombocytopenic purpura (ITP). Several systemic reviews have recently been performed and this effect had been confirmed. Meanwhile, few reports regarding the long-term effects of eradication are available. *Aims.* We present 8-year follow-up data for patients with ITP considered to be related to H. pylori infection, which was eradicated successfully. *Methods.* Patients diagnosed as having ITP at the Tokyo Metropolitan Komagome Hospital and who had been involved in a study evaluating the effects of H. pylori infection on ITP in 2001 were included. After infection was confirmed, eradication was performed and their platelet counts were followed. They were re-evaluated in 2009 for H. pylori infection and platelet count. Medical records were also examined for platelet counts in the interim. H. pylori infection was examined using 13C urea breath test (UBIT; OTSUKA Pharm. Co., Ltd, Tokyo, Japan). Eradication of H. pylori was performed using standard therapy: amoxicillin (1,000 mg, twice daily), clarithromycin (250 mg, 3 times daily) and proton-pump inhibitor (20-40 mg, twice daily) for 1 week. *Results.* In 2001, 31 patients with chronic ITP were evaluated for H. pylori infection. Nineteen of these were found to be positive and were treated for H. pylori infection. Eight years later, 13 of these 19 patients were re-evaluated for H. pylori and platelet count was followed in 17 of 19. Two patients were lost during follow-up at 5 years after treatment. At the time of eradication treatment, mean age was 59.7 years (29-74 years), 8 patients were male and median length from diagnosis was 9.3 years (0-28 years). Nine patients had received steroid treatment and splenectomy had been performed in 3. Before eradication, platelet count exceeded 100 $\times 10^9$/L in 2 patients, partly because of steroid treatment, and their platelet

counts remained below the normal range after eradication. Therefore, these patients were excluded from further discussion. Initially, 6 of 17 patients did not respond to treatment and remained no response after eight years. The platelet counts of the remaining 11 patients were initially below 50×10^9/L and 10 patients showed complete response within 6 months after treatment for H. pylori, and this was sustained for 8 years. Of these, one patient was lost during follow up after 5 years, when normal platelet count was documented. Steroid treatment was discontinued in all three patients and no relapse was observed or reported. Two of the 12 patients examined for H. pylori infection after 8 years were positive, but the timing of infection was unknown. *Conclusions.* At our institution, 11 patients who responded to treatment remained free from thrombocytopenia and no relapse has been reported during the last 8 years after treatment. Due to the difficulty of following elderly patients, the number of patients in present study is small. However, the information presented here remains valuable in establishing the long-term follow-up strategies for these patients.

1955

COMPARATIVE STUDY OF THYROID HORMONES WITH THE NUMBER, MEAN VOLUME AND DISTRIBUTION WIDTH OF PLATELETS

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Background-Aim. To study comparatively the possible correlation of the thyroid-stimulating hormone (TSH), thyroxine (T4) and triiodothyronine (T3) with the plain platelet parameters of a blood test (mean platelet volume (MPV), platelet distribution width (PDW) and platelet number), in cases of euthyroid patients and hyperthyroid patients. *Material-Method.* The lab test results of 75 euthyroid patients aged from 25 to 75 years (16 male and 59 female, with average age 56 years) and 46 hyperthyroid patients with similar age (12 male and 34 female, with average age 54 years) were studied. The values of the thyroid hormones were determined with the use of an automated hematogical analyzer in all the patients, followed by a statistical analysis of the data with the use of the SPSS v.10 statistical packet. *Results.* As far as T3 and T4 are concerned, there was no statistically important correlation with any of the platelet parameters, in both euthyroid and hyperthyroid patients. (In the hyperthyroid patients there clearly was a greater correlation from the euthyroid patients, but it too was not statistically important, $P>0.05$).On the contrary, as far as TSH is concerned, there was a statistically important negative correlation ($P<0.05$) with MPV and PDW in both euthyroid and hyperthyroid patients. The correlation exhibited was greater in the hyperthyroid patients (correlation coefficient r= -0.698 for MPV and r= -0.845 for PDW) and smaller, but statistically important, for the euthyroid patients (correlation coefficient r= -0.653 for MPV and r= -0.811 for PDW). More specifically, as shown in both groups, this negative correlation was stronger for PDW, compared to MPV. *Conclusions.* It is proven, therefore, that the low value of TSH is related with less in number and greater in volume activated platelets, who surely play an important role in the coagulation and endothelial disorders taking place in hyperthyroid patients.

1956

RITUXIMAB IN THE TREATMENT OF IMMUNE THROMBOCYTOPENIA: FOLLOW-UP IN SEVEN CASES

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Background. Rituximab is a monoclonal antibody against the CD20 antigen whose safety and efficacy in the treatment of children with chronic immune thrombocytopenia has been demonstrated. It can be used in cases not responding to splenectomy and as an alternative to this procedure. Nevertheless, experience of its use in children with immune thrombocytopenic purpura (ITP) is limited, the largest cohort including 49 children and the longest follow up reported of 39 months. *Aims.* To report the experience of our center with the use of rituximab in children with severe chronic immune thrombocytopenia requiring repeated courses of corticosteroids and immunoglobulin. *Methods.* Retrospective study including 7 children with chronic ITP treated with rituximab in a pediatric hematology unit between December 2006 and August 2009. Data were collected from clinical records and included demographic data, treatments and blood results before rituximab therapy, response to therapy, duration of remission and adverse effects or treatment failure. *Results.* We treated 7 children now aged 4 to 18 years old. Previous ITP duration was in average 27 months (range 4 to 72 months). All children had been previously treated with steroids and immunoglobulin and one had been splenectomized. Complete response was observed in 3 children and sustained in 2 of them after 32 e 36 months follow up time. The other responding child had an initial partial response (platelet count range: 50-150.000/μL) for12 months followed by complete response for 7 months and had then a recurrence during a viral illness (influenza A). Partial response occurred in one more child and minimal or absent response was observed in 3 children. Responses occurred in all but one case after the first or second doses. Two patients were subsequently submitted to splenectomy for persistent severe ITP. Follow up time ranged from 3 to 32 months (average 22.5 months). Mild adverse effects were registered in 3 patients, consisting in pruritus, urticaria and vomiting. They all occurred during the first dose and resolved with symptomatic therapy or with reducing the infusion rate. Therapy was continued in all cases. *Summary/Conclusions.* Rituximab is a promising treatment option for children with severe treatment refractory chronic ITP. It seems to provide a sustained response in responders with minimal and tolerable toxicity. It may provide an alternative to splenectomy, especially in younger patients who have increased risk of serious infections with encapsulated organisms.

1957

ANTI-D IMMUNOGLOBULIN A COST-EFFECTIVE ALTERNATIVE FOR IMMUNE THROMBOCYTOPENIA

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Background. Anti-RhD immunoglobulin (anti-D) is a therapeutic alternative successfully used in the setting of both acute and chronic immune thrombocytopenia. Anti-D therapy appears to inhibit macrophage phagocytosis by a combination of both FcR blockade and inflammatory cytokine inhibition of platelet phagocytosis within the spleen. The largest body of evidence exists for the competitive inhibition of the mononuclear phagocytic system by sensitizes red blood cells. Evidence to support this as a major mechanism of the therapy includes studies which show that anti-D is ineffective in Rh-D negative patients and relatively ineffective at standard doses in patients who have had a splenectomy. *Aims.* Previous theoretical cost-minimization models have demonstrated the cost-benefit of administering Anti-D rather than IGIV for the treatment of patients with ITP. Intramuscular administration of anti-D seems to offer the same efficacy as intravenous administration but with fewer side effects. Here we report our experience with intramuscular anti-D for patients with immune thrombocytopenia (ITP). *Methods.* The clinical study comprise a group of 7 patients, aged between 2 years and 4 months and 30 years(mean 11,8 diagnosed with immune thrombocytopenia: 2 with acute and 5 with chronic evolution. There have been included only RhD positive and HIV 1-2 negative patients, requiring treatment for clinical manifestations and/or platelet count under 30×10^9.The route of administration was intramuscular for 3 consecutive days at initial dose of 50 microg/kg. The platelet daily value was followed concomitant with the level of hemoglobin, billirubin, serum urea, hemoglobinuria and parameters of coagulation. *Results.* In 6 cases of the group(85,7%), anti-D immunoglobulin showed efficacy after 7 days of the first administration with an increase in platelet count greater than 40×10^9/L (extreme values: $53\text{-}214\times10^9$/L) in condition of decrease in hemoglobin level of maximum 4 g/l only in 1 patient.The response in 4 cases was good with the correction of symptoms during 10-14 days. In two cases, after thrombocyte count restoring, splenectomy was performed, with a complete response. The maintenance therapy consisted in repeated doses of anti-D immunoglobulin weekly at doses of 10-25 microg/kg and/or in condition of trombocyte count below 30×10^9/L. Pain at the injection site was common but self-limited with no effect on activity level. Headache was reported in 3 cases, digestive complains in 5 cases, mialgia in 3 cases and local allergic rash in one case.Severe side effects were not noted. Despite of the small number of patients the results are encouraging. *Conclusions.* The use of anti-D immunoglobulin showed benefits in symptoms control of ITP in adults and children. Anti-D therapy has been shown capable of delaying splenectomy in adult patients,

but does not significantly increase in total number of patients in complete remission. The low grade toxicity and 50-60% reduction of costs in comparison to IVIG recommends the anti-D therapy. The treatment of ITP patients with Anti-D seems to provide cost-savings compared to treatment with IVIG.

1958

EFFICACY AND SAFETY OF SUCCESSFUL TREATMENT IMMUNE THROMBOCYTOPENIC PURPURA (ITP) WITH THE THROMBOPOIETIN-MIMETIC ROMIPLOSTIM

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Background. Immune thrombocytopenic purpura (ITP) is a relatively common autoimmune disorder characterized by the production of antibodies against circulating platelets. Symptoms can be mild, but several patients are in high risk for bleeding and require treatment. Glucocorticoids followed by spleenectomy had been the mainstays of therapy. Recent findings suggest that immune thrombocytopenic purpura (ITP) is not only due to increased platelet destruction by to anti-platelet antibodies but there is also defect in the production of platelets by megakaryocytes. Thrombopoietin (TPO) is the normal regulator of thrombopoiesis. Romiplostim is a second-generation thrombopoietic receptor agonist that exerts its therapeutic effect by stimulating megakaryopoiesis and thrombopoiesis. *Aim.* We studied the efficacy and the safety of treatment immune thrombocytopenic purpura (ITP) with the thrombopoietin-mimetic Romiplostim. *Patients and methods.* We describe 4 patients [median age 48 years (19-62)], 3 women (2 with splenectomy) and 1 man, with chronic immune thrombocytopenic purpura refractory to other therapies, including splenectomy that is in successfully managed with romiplostim. The median time of follow up is 60 months (108-36 months). The diagnosis in our patients relies on the exclusion of alternative disorders because no specific criteria establish the diagnosis of ITP. Additional laboratory tests provide no evidence of secondary thrombocytopenia, suggesting a diagnosis of ITP according the American Society of Hematology (1). For all patients first choice of therapy is the corticosteroid. The first patient, for relapse after splenctomy received corticosteroids with satisfactory results but the treatment stopped because of psychiatric side effects. Then, she received danazol, cyclophospamide and azathioprime with poor results. At last, for 5 months she was in therapy with intravenous immune globulins with satisfactory results. Finally, two years after splenctomy, the last 40 weeks, the patient is in a weekly therapy with romiplostim 4_g/kg. The second patient for relapse after splenctomy received Romiplostim in combination to cyclosporine for 10 weeks until now. Two others patients refused splenectomy, they had serious side effects for corticosteroid and received Romiplostim 1 μg/kg for the last 8 weeks. *Results.* Initially all patients received Romiplostim 1 μg/Kg subcutaneous but everyone need dose adjustments to maintain normal or satisfactory platelet counts. All patients have managed to maintain a platelet count of above 50×10^9/L with no severe adverse-events and with no hemorrhagic events. Regarding side effects the patients had mild headache with duration of 24 hours after the Romiplostim administration that receded with paracetamol. Romiplostim changed patient's life style improving the quality of their life, although it was not able to cure the disease. *Conclusion.* The thrombopoietin-receptor agonists, with their first representative Romiplostim, give a new prospect of treatment in chronic and refractory disease. Romiplostim is very effective, has a low number of side effects, excellent tolerance and improves quality of life. Although, it doesn't offer cure of the disease, it offers hope and safety for patients with chronic immune thrombocytopenic purpura.

1959

HEMATOLOGICAL FEATURES - AND PARTICULARLY PLATELET STATUS - IN PREECLAMPSIA

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Background. The hematological picture of preeclampsia varies from normal laboratory tests to severe thrombocytopenia (due to the major role of platelets in pathophysiology, platelet activation and consumption), anemia, reactive leucocytosis/leucopenia. *Aims.* To study the hematological alterations of patients with preeclampsia, with a special accent on platelet function. *Methods.* Transversal study of 30 patients with preeclampsia, *vs.* 30 controls, from University Emergency Bucharest and Elias Hospitals, Romania. We studied clinical and laboratory parameters and platelet function was assessed via flowcytometry techniques - markers of activation (CD62P: P-selectin, CD63: granulophysin), aggregation (CD41/CD61), adhesion (CD42b/CD42a, CD31 - platelet endothelial cell adhesion molecule). *Results.* 25/30 patients (83.3%) had a severe form of preeclampsia; 17/30cases (56.6%) associated intrauterine growth restriction (IUGR). Two patients (6.7%) evolved to eclampsia; HELLP syndrome complicated 6/30cases (20.0%). In one case, therapeutic abortion was performed at 22weeks of gestation. All pregnancies ended with C-section (at 35.6weeks of pregnancy in medium). From 29 newborns (medium weight 2332 grams), 13 (44.8%) were prematures, 6 (20.7%) were small for gestation age; 2 premature newborns died after 48hours (pulmonary hemorrhage), respectively 2weeks (intraventricular hemorrhage, sepsis with Klebsiella). Regarding laboratory parameters, CBC revealed: medium-severe anemia in 9/30patients - 30.0% (5 having HELLP syndrome with hemolytic microangiopathic pattern), slight leucocytosis in patients (medium WBC 11318/mmc *vs.* 10578/mmc) and lower platelet count [181800/mmc - patients *vs.* 247172/mmc - controls (P=0.01)], with a significantly higher medium platelet volume in patients - 11.3fl *vs.* 8.7fl, P<<0.0001; 12/30 (40.0%) patients presented thrombocytopenia. Biochemistry showed only increased liver enzymes in patients: medium AST 312.1U/l *vs.* 20.5U/l; ALT 126.2 *vs.* 22.0U/l; LDH was also elevated (twice the normal value). Patients presented higher platelet activation status: medium CD62P expression 74.9% in patients with preeclampsia, *vs.* 15.9% - controls (P<<0.0001); CD63: 27.7% - patients *vs.* 8.4% - controls (P<<0.0001); CD31 was also significantly higher in patients with preeclampsia: 94.1% *vs.* 68.6% (P=0.0001). For adhesion and aggregation markers, the results were different - for CD42a and CD61: results are comparable [CD42a: 95.3% - patients and 90.8% - controls (P=0.05)], [CD61: 99.2% - patients and 98.6% - controls (P=0.14)]; for CD42b and CD41, medium values were higher in patients *vs.* controls: [CD42b: 93.2% - patients and 72.5% - controls (P<0.05)], [CD41: 82.9% - patients and 68.3% - controls (P=0.02)]. We also searched for significant correlations among studied parameters; we interestingly observed: negative correlation between platelet count and activation status expressed through CD62P (r=-0.657), positive correlation of systolic BP with activation marker CD62P (r=0.846) and with aggregation marker CD41 (r=0.648), and also a positive correlation between MPV and CD31 (r=0.637). *Conclusions.* preeclampsia is a severe pregnancy complication which may pose important consequences for mother and fetus - in our study: over 50% incidence of IUGR, 45% premature newborns, 6.7% newborn deaths. Laboratory picture of preeclampsia associates thrombocytopenia (which may become severe), but with younger, regenerative platelets present (higher MPV). Platelets of preeclamptic patients are significantly more activated and express a higher level of adhesion to the endothelium; the level of activation rises with the decrease of platelet count and with the increase of blood pressure.

1960

CHANGE IN CD4/CD8 RATIO AND ABSOLUTE NEUTROPHIL COUNT AFTER INTRAVENOUS IMMUNOGLOBULIN INFUSION IN PEDIATRIC PATIENTS WITH IMMUNE THROMBOCYTOPENIC PURPURA

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Background. high doses of Intravenous immunoglobulin (IVIG) treatment of patients with autoimmune thrombocytopenic purpura influence lymphocyte number and function. In this study we assessed effect of Intravenous immunoglobulin treatment on neutrophil and lymphocyte count, CD4 /CD8 ratio in patients with ITP. *Materials and methods.* In this study, we analyzed blood sample in 32 patient with ITP before and 1h after completion of IVIG infusion. In all patients platelet, white blood cell and differential count performed before and after IVIG infusion. The following, lymphocyte phenotypic markers CD4 and CD8 lymphocytes were also examined. Statistical analysis was carried out using paired t-test. Correlation between age and total volume IVIG infused with variables analyzed with Pearson coefficient correlation. *Results.* Cellular blood count show significant decrease in leukocyte (P<0.001), neutrophil (P<0.001) and lymphocyte count (P<0.001) 1h

after IVIG infusion, but this changes was not significant in number of platelets (P=0.377). CD4/CD8 ratio increase significantly after IVIG infusion (P<0.001). percent of CD8 lymphocytes decrease significantly 1h after IVIG administration (P=0.001). absolute count of CD4 and CD8 lymphocytes significantly decrease after IVIG treatment. (P<0.001) *Discussion.* It seem that the IVIG decrease Absolute Neutrophil Count (ANC) after treatment and can be causes of decrease in lymphocytes number. Probably change in CD4 and CD8 lymphocytes dependent to time of sample collection after IVIG infusion.

1961

STUDY OF THE CHANGES OF COAGULATION FACTORS DURING PREGNANCY

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Background-Aim. To investigate the changes of the factors related to the coagulation mechanism during pregnancy and to estimate the magnitude of hypercoagulation that they cause. As it is already known, from the 2nd trimester onwards there is an increase of both fibrinogen and antithrombin III while fibrinolysis and protein C decrease and so does the number and efficacy of platelets due to the increase of prostacyclin. *Material-Methods.* In our study 72 parturients were included who, among other lab tests, were tested from the 2nd trimester of pregnancy onwards with complete blood count as well as tests related to blood coagulation including prothrombin time (PT), partial thromboplastin time (PTT) and also fibrinogen (FIB). *Results.* PT<12 sec was found in 34 women (47.2%), PTT>40 sec in 11 women (15.3%), while FIB>4 g/l was found in 63 women (87,5%) of whom 11 (15.3%) had FIB> 6 g/l. Lastly, in regard to platelets, in 12 cases (16.7%) a platelets number lower than 140,000 was found - this was certified by microscopy examination of peripheral blood films - and in almost all cases, a decrease was recorded in following check-ups as pregnancy progressed. *Conclusions.* Consequently, it is proven that in many cases (42.7%) PT decreases during pregnancy, while in fewer cases (15.3%) an increase of PTT is recorded. Also, in the vast majority of cases (87.5%), fibrinogen is increased - and in some of them significantly increased -, while often there is a low platelets number, that continues to decrease as pregnancy progresses. Therefore, there is a great need of conducting the tests necessary to evaluate the coagulation mechanism in every pregnant woman - the earlier the better - and particularly in high risk women.

1962

STUDY OF THE CHANGES IN FIBRINOGEN AND CRP LEVELS IN ACUTE MYOCARDIAL INFARCTION AND CHRONIC LUNG OBSTRUCTION

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Background-Aim. To describe the changes in fibrinogen and CRP levels in patients with acute myocardial infarction(AMI) and with exacerbation of chronic lung obstruction and to evaluate the changes in these two conditions, both in comparison with the normal ranges and between the two groups of patients. *Material-Methods.* We studied 42 patients with AMI (33 men and 9 women) and 31 patients with exacerbation of chronic lung obstruction (18 men and 13 women) who were admitted in the Cardiology and Internal Medicine Department.For fibrinogen level measurement a Multifibren U reactive of DATE BEHRING Company was used while CRP levels were measured with a biochemical analyzer. For statistic evaluation of the results we used the statistic system SPSS v 13. The normal range for fibrinogen was 150-450 mg/100 ml and for CRP < 0,5 mg/dL. *Results.* a) The fibrinogen levels in patients with AMI were lower than normal range in one patient (2,4%), within normal range in three patients (7,1%) and higher than normal range in 38 patients (90,5%). Regarding to fibrinogen levels in patients with exacerbation of chronic lung obstruction we didn't find any patient with levels lower than normal range (0%), one patient within normal range (3,2%) and 30 patients higher than normal range. b) Regarding with the CRP levels in patients with AMI, we didn't find any patient with levels lower than normal range (0%), 10 were within normal range (23,8%) and 32 higher than normal range (97,6%). In patients with exacerbation of chronic lung obstruction we found no patients with lower than normal CRP levels, 3 patients within normal range (9,7%) and 28 with higher than normal range levels (90,3%). The correlation between fibrinogen and CRP was 0,21 in the AMI group and 0,5 in the chronic lung obstruction group. The average for fibrinogen was 572 in the AMI group and 632 in the chronic lung obstruction group (P=0,2).The average for CRP was 7,65 in AMI group and 8,15 in the second group (P=0,12). *Conclusions.* In the chronic lung obstruction group the fibrinogen and CRP levels were higher than normal in 96,8% and 90,3% of the patients while in the AMI group the percentages were 90,5% and 76,25. The correlation between fibrinogen and CRP was 0,5% in the chronic lung obstruction group while in the AMI group is much lower than 0,21.

1963

THE ROLE OF MULTIDRUG RESISTANCE PROTEIN(MRP-1) IN PLATELET ACTIVATION WITH ALLERGIC ASTHMA

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MRP-1 may play a role in the pathology of alergic asthma. It is known that LTC4 which is the substrat of MRP-1, activates the inflamation in asthma.However ,the role of MRP-1 in platelet activation and the platelet-leucocyte association still remain unclear. There fore, we planed to investigate the gene expression level of mRP-1 in leucocyte of adult subjects with allergic asthicmatic patients (26) and explore preliminarily in correlation between mRP-1 expression level and the platelet activation. Quantitative real time PCR studies have demonstrated that MRP-1 expression levels were increased in leucocytes ofmild persistant asthmatic patient which is compared to control. We also evaluated the existence of possible association through a flow cytometry analysing the platelet-leucocyte agregates considered as an effective method to define activated platelets. We found that the CD42b fluorescent percentage, has been found significantly higher in asthmatic patients which has high level of MRP-1 expresion than the control group. We found positive correlation between MRP-1 expression levels and platelet activation with severity of asthma.

1964

VARICONZOLE HAS NO EFFECT ON PLATELET FUNCTION

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Background. Voriconazole is a recent triazole with broad-spectrum antifungal activity against clinically significant and emerging pathogens. It is widely used for life-threatening opportunistic fungal infection. Despite an extensive safety database, we could not know whether it might produce functional influence on platelet response. *Aim.* In this study, the effect of voriconazole on platelet aggregation was investigated. *Methods.* Platelet function tests were performed in 16 healthy male volunteers. Three concentrations of voriconazole solution were prepared that would result in 1, 2, 4 and 8 μg/mL voriconazole concentrations in the blood similar to that observed after clinical therapeutic oral or intravenous application. Each concentration of voriconazole solution and a control diluent without voriconazole were incubated with whole blood at 370C. After incubation for 15 min, aggregation responses were evaluated with adenosine diphosphate (ADP) (5 μM) and collagen (2 μg/mL) in platelet-rich plasma. *Results.* When compared to control, preincubation with all dilutions of voriconazole have no effect on platelet aggregation response induced by ADP and collagen in a statistically significant manner (P>0.05 for all comparisons). Voriconazole showed also no effect on platelet aggregation in a dose-dependent manner. *Conclusion.* This study with an *in vitro* model showed that voriconazole administration in clinically therapeutic doses had no effect on platelet aggregation.

1965

BLEEDING AFTER CIRCUMCISION IN EGYPTIAN PATIENTS WITH HEMOPHILIA A: A FIVE YEAR PROSPECTIVE STUDY OF TWO PEDIATRIC HEMOPHILIA CENTERS

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Background. Circumcision is a risky practice for hemophilia A patients especially in the developing countries, due to Lack of adequate factor supply. *Aims.* To evaluate the incidence of bleeding after circumcision in Hemophilia A patients in spite of planned management and to report the deviation from that planned treatment. *Methods.* One hundred and sixty patients with hemophilia A aged 1 to 17 years (median = 8 years) who had performed circumcision in our center between 2004 and 2009 were followed up. The hospitalization was planned to be two-three days and factor replacement therapy seven to ten days. All were inhibitor negative before circumcision; inhibitor development among them was reported / 6 months. *Results.* Hemophilia A was mild in 32(20%), moderate in 32(20%), and severe in 96(60%). Median age for circumcision in the first child with no positive family history was 2 months while in subsequent siblings was 6 Years. Positive family history was recorded in 60%. Ten percent of children developed inhibitors (4% low titer and 6% high titer) 2-12 months following circumcision and were treated with low dose immuno-tolerence. Planned therapy included 40% cryoprecipitate, 60 % factor VIII plasma derived and 25% received tranexamic acid, while 100% of those > 1 year underwent circumcision under general anesthesia. A deviation from planned post operative replacement therapy was 20% prolonged and 10 % reduced while 10% had mild bleeding and only one patient developed uncontrolled bleeding and received VIIa 90 unite/kg 2 doses and was controlled .Wound re-suturing in 5 % , the stay in hospital was prolonged > 2 weeks in 2 patients. No deaths were reported during the study period. *Summary/Conclusions.* Most of circumcised hemophilia A were treated as planned with low incidence of mild bleeding. Ten percent developed FVIII inhibitors and VIIa was used only occasionally in uncontrolled bleeding.

1966

A SENSITIVE & SPECIFIC CHROMOGENIC ASSAY FOR THE DETECTION OF 'FUNCTIONAL FVIII INHIBITORS'

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Upto 25-30% of severe Haemophilia A patients and ~5% of mild or moderate Haemophilia A patients develop alloantibodies or "FVIII inhibitors"against the infused FVIII. The Bethesda assay has been the classical laboratory assay for the quantitation of inhibitory antibodies to FVIII since first published by Kasper *et al.* in 1975. Various modifications and newer methods like ELISA with increased sensitivity and specificity for the detection of inhibitors have been reported. However, in spite of the increased sensitivity achieved, the quantitation of FVIII antibodies still involves important unresolved issues such as inter-assay variability, lack of appropriate standardization of the lower limit of inhibitor detection and the definition of a negative antibody titre, as well as interference by non-neutralizing antibodies. Hence, methods to detect very low-titre FVIII inhibitors on the rise or the detection of FVIII antibodies without interference from non-neutralizing antibodies (lupus anticoagulants and other interfering antibodies) are still being explored. We report here a sensitive and specific chromogenic assay using a Coamatic Factor VIII. (Chromogenix) using a substrate S-2765 which detects only the functional FVIII inhibitors. The colour is then read photometrically at 405 nm. We tested two inhibitor positive samples and the assay was found to be sensitive upto 0.1 Bu/mL of inhibitor levels which is much lower than that detected by the conventional Bethesda assay (0.5 Bu/mL). The total time required for the detection is 2.5 hours which is much less than that of Bethesda assay. Detection of very low titer of factor VIII much earlier has specific advantages in that the therapeutic management can be modified much earlier before it can be detected by the conventional Bethesda assay. This will have also its implications as a screening technique prior to surgery as in India there is a high prevalence of patients developing inhibitors following surgery.

1967

THROMBIN GENERATION AND OTHER COAGULATION MARKERS IN PATIENTS WITH LIVER CIRRHOSIS

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Background. The role played by coagulation defects in the occurrence of bleeding in cirrhosis is still unclear. Conventional coagulation tests (PT/INR and aPTT) seem unable to predict the severity of bleeding problems in patients with cirrhosis of the liver possibly because they do not adequately reflect the balance between procoagulant and anticoagulant clotting factors. Recently a test has become available to routinely measure the endogenous thrombin generation potential (ETP) by Dade Behring (Marburg, Germany). *Aim.* The comparison of ETP values and other coagulation markers between controls and patients with liver cirrhosis. *Methods.* 56 samples, of consecutive patients with histologically confirmed liver cirrhosis, and 30 samples of controls were investigated for PT/INR, fibrinogen, d-dimers and ETP parameters. We used the chromogenic method on the fully automated Behring Coagulation System (BCS) for the measurement of thrombin generation parameters. *Results.* 6 patients had alcoholic cirrhosis, 22 HCV, 5 PBC, 7HBV, 1HBV and HDV and 13 cirrhosis of unknown origin. Table. *Summary/Conclusions.* The automated ETP test can play an important role in the evaluation of haemostatic liver function in patients with cirrhosis. A potential clinical implication of these findings is that the laboratory investigation of the coagulation function, presently performed with the PT and APTT, may be inadequate to assess the true risk of bleeding when patients with cirrhosis undergo invasive procedures such as liver biopsy and transplant surgery. Perhaps the measurement of thrombin generation might be more suitable to evaluate the hemorrhagic risk. Although plausible, this hypothesis needs to be sustained clinically by a prospective study.

Table.

	CONTROLS		PATIENTS		
	mean	sd	mean	sd	p
tlagsec	19.3	2.6	21.5	8.1	0.16
tmax sec	54.3	4.4	84.4	89.6	0.015
CmaxmAmin	123.5	6	78	23.4	0.0001
ETPmA	394.7	29.5	284.4	73.2	0.0001
INR	0.9	0.06	1.3	0.3	0.0001
FIBmgldl	403.9	110.3	310.4	138.7	0.008
DDmgll	0.27	0.15	2.3	5.2	0.049

1968

ACQUIRED COAGULOPATHIES: NINE CLINICAL CASES

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Introduction. Acquired coagulation factor inhibitors are circulating immunoglobulins that inhibit the procoagulant factor activity. Extremely rare, result from an immune impairment, and can be associated with different antibody types: auto, allo and xenoantibodies. Autoantibodies arise spontaneously in patients without prior coagulopathy and are associated with autoimmune diseases, cancer, infections, pregnancy, drugs and bone marrow transplantation. In many cases their aetiology is unknown, and their presence is frequently associated with catastrophic bleeding. The lupus-like anticoagulants are the most prevalent. The ones that cause more severe bleeding are directed to a specific factor (F), most frequently FVIII. Laboratory diagnosis is suspected by prolonged PT and/or aPTT and low circulating levels of a particular clotting fac-

tor, in a patient without prior bleeding history, and confirmed by titration of the inhibitor. The main therapeutic purposes are controlling the bleeding events, treating the cause and suppressing the antibody. *Main goal.* To describe 9 clinical cases of acquired coagulopathy (AC) in order to illustrate the importance of an early diagnosis and aetiological identification. *Clinical cases.* 9 patients with AC diagnosed in the CHC Hematology Department: 1 with anti-prothrombin antibodies; 2 with FV inhibitors; 3 with acquired FX deficiency; 1 with acquired von Willebrand disease and 2 with acquired A Hemophilia. Patients' median age was 68 years; 3 female and 6 male. Severity of bleeding: 3 major, 5 minor and 1 asymptomatic (diagnosed during a preoperative investigation of a prolonged aPTT). Bleeding events were treated with local haemostatic measures, antifibrinolytic and bypass agents, with efficacy in all cases, except for 1 in which bleeding was controlled, but, at day 12 of admission, the patient died with a Central Nervous System haemorrhage. It was only possible to determine the AC aetiology in 5/9 patients. In 1/9 the AC appeared during an infection and was associated with an anti-prothrombin lupic anticoagulant, which disappeared spontaneously with infection resolution. Three patients had conditions that have been linked to the emergence of AC: 1 amyloidosis, 1 Waldenstrom's macroglobulinemia and 1 antiphospholipid syndrome. The inhibitors disappeared after the underlying condition treatment. In 1 patient the AC was due to an acquired FX deficiency related with valproate. There was normalization of FX plasma levels after withdraw of the drug. *Conclusion.* 8/9 patients presented with bleeding. Laboratory screening for inhibitors was positive in 5/9, and negative in the acquired FX deficiencies (3) and vWF (1). Whenever was possible to treat/eliminate the AC aetiological agent (5), the specific F plasma levels normalized. An AC should be suspected in patients without prior hemorrhagic history presenting sudden haemorrhage and prolonged PT and/or aPTT. The search for inhibitors should be done immediately as well as an exhaustive search of an aetiological factor, because the rapid diagnosis is crucial for an early correct treatment.

1969

A SEMIQUANTITAIVE ASSAY FOR DIAGNOSIS OF FACTOR XIII DEFICIENCY

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The most widely used test in routine haemostasis laboratories for factor XIII deficiency is the clot solubility test in which the plasma is clotted either with thrombin or Calcium chloride and resistance to lysis is observed either with urea or monochloro acetic acid. Though these methods are highly specific they can detect only severe factor XIII deficiency i.e upto <2 U ML -1 leaving mild to moderate factor XIII deficient cases undiagnosed. In the present study we have compared the sensitivity of different clot solubility tests in the diagnosis of factor XIII deficiency. We used both 0.025M CaCl2 and 10u/mL thrombin for forming the clot and used 5M Urea, 1% monochloroacetic acid and 2% acetic acid for dissolving the clot and the solubility was observed after 24 hours.It was observed that the combination of 1% monochloroacetic acid plus thrombin/CaCl2 and 5M urea plus thrombin/CaCl2 is specific for factor XIII deficiency but the detection limit was upto 1 U ml-1.The best combination was found to be2% acetic acid with 10u/mL thrombin where the detection limit was upto 12.5 U ml-1. We conclude that performing a two tube method i.e 5M Urea/1% monochloroacetic acid plus CaCl2 and 2% acetic acid plus 10U.ml thrombin we should be able to differentiate between mild and severe factor XIII deficiency which otherwise is not possible by the conventional assays presently being followed in all the laboratories. Using these two sets of reagents, we can detect factor XIII level in three ranges i.e <1%, 1-12.5% and >12.5%.

1970

A STABILITY MONITORING AND EASTBLISHMENT FOR FACTOR VIII: CONCENTRATE

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Background. In therapeutic use, the dosage of Blood coagulation factor VIII product is determined depending on the concentrate in blood, and it is essential to measure the potency accurately. Establishment of standard is required for measuring the potency. The current Korean standard for Factor VIII Concentrate, established in 2001 for measuring the potency, showed decreasing trend according to 'Establishment of Quality Management System and Evaluation Techniques of Stability Testing for National Biological Standards' in 2008. A multicenter study was carried out to evaluate the suitability of a candidate as the 2nd Korean standard and the stability. *Aims.* It is to establish 2nd Korean standard and analyze the stability trend for contribute the globalization of quality control in blood coagulation factor VIII product by establishing the standard. *Method.* Before the study, the stability trend analysis of 1st Korean standard was All participants performed 24 independent assays with an exception of one laboratory performed 35 assays for clotting and 29 assays for chromogenic. Each participant was requested to perform both two assays; the clotting assay and the chromogenic assay. Each assay result was analyzed for its accuracy, precision, linearity and specificity. *Result.* The estimated geometric mean value of potency differed from each assay; 8.9 IU/vial in clotting assay and 7.4 IU/vial in chromogenic assay. Each assay method was used to measure each potency. *Summary/Conclusion.* Based on the result, the candidate standard is accepted as the 2nd Korean National Standard for factor VIII:C concentrate. In addition, a continuous monitoring of stability studies for the 1st and 2nd Korean standard is in progress.

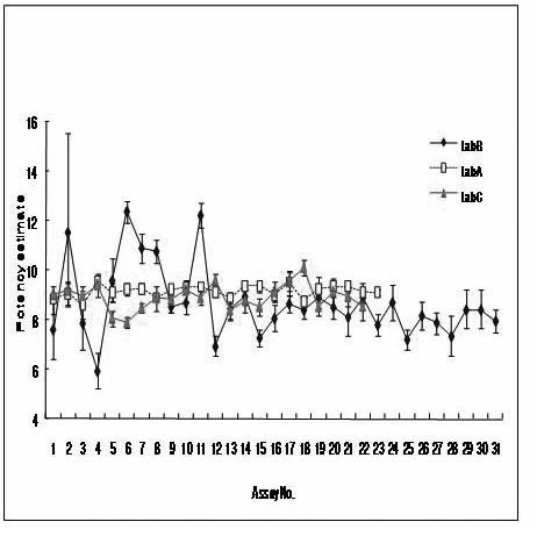

Figure 1. Distribution of potency using one-stage clotting assay.

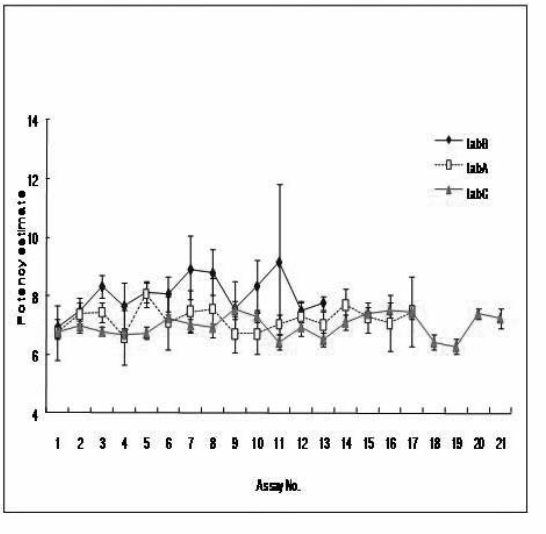

Figure 2. Distribution of potency using chromogenic assay.

1971

SUCCESSFULL USE OF PROPHYLACTIC RECOMBINANT FACTOR VIIA IN A PATIENT WITH CONGENITAL FACTOR VII DEFICIENCY WITH INHIBITORY ANTIBODY

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Background. Congenital factor VII (FVII) deficiency is a haematological disorder characterized by increased bleeding tendency in the affected patients. Bleeding episodes seen in these patients are treated with recombinant factor VIIa (rFVIIa). The inhibitory antibody formation could be seen in patients with severe FVII deficiency because of recurrent FVII replacement theraphies. A few publications have appeared on treatment of congenital FVII-deficient patients with inhibitory antibody until so far. *Case report.* The patient reported here is 3-month-old male patient. He was admitted to our clinic because of intracranial bleeding. FVII deficiency caused by a homozygous nonsense mutation (p.Ser112Stop (c.335C>G)) on FVII gene was detected (FVII:C %2). The patient exposed to recurrent bleeding attacks including epistaxis, intracranial, gastrointestinal, cutaneal and mucosal bleedings. rFVIIa was used succesfully during the bleeding episodes. When the patients was 1 year old, inhibitory antibody became positive (32 Bedhesda units). Althought the persistancy of inhibitory antibody to FVII, classical dose rFVIIa (20-40 mcg/kg/dose) was used effectively. Because of recurrent intracranial bleedings, the prophylactic rFVIIa (25 mcg/kg/dose, 3 dose weekly) were used. No serious bleeding episodes including intracranial bleeding were seen and the frequency of minor bleedings were appearently decreased within 12 months of prophylaxia. *Conclusion.* p.Ser112Stop (c.335C>G) mutation detected in our patient is known to cause severe FVII deficiency. Probably, the inhibitory antibody to FVII has occured because of recurrent rFVIIa replacement theraphy. However, it may be a non inhibitory antibody, because classical replacement dose of rFVIIa is effectively used for bleeding episodes. Althought the severe FVII deficiency and inhibitory antibody are present, prophylactic rFVIIa (25 mcg/kg/dose, 3 dose weekly) were used successfully. There are few data for prophylaxis in congenital FVII deficiency. The use of prophylactic rFVIIa may be effective in patient with FVII deficiency and inhibitory antibody.

1972

MOLECULAR PATHOLOGY OF HAEMOPHILIA A IN INDIAN PATIENTS

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Haemophilia A (HA), caused due to factor VIII deficiency, is an inherited X-linked disorder, affects in 1 in 5000 males crossing all economic, geographical and racial boundaries. The factor VIII gene is 186 Kb long having 26 exons and 25 introns. The mutations in the factor VIII gene are highly heterogeneous in nature and almost 1/3rd of the mutations can be *de novo*. Approximately 50% and 2-5% of severe haemophilia A patients are positive for the two recurrent mutations i.e intron 22 and 1 inversions respectively. We report herewith mutation analysis in 41 inversion negative HA cases (32 unrelated and 9 familial) by multiplex PCR and CSGE technique followed by sequencing to confirm the mutation. We had 10 severe (FVIII:C <1%), 14 moderate(F8 1-5%), 8 mild (F8 5-50%) cases. 12 missense, 7 deletions, 2 insertions, 1frame shift, 4 nonsense mutations, out of which 17 were recurrent mutations and 10 were novel. The novel substitution mutations were found to be deleterious using the different prediction softwares. We also encountered a double mutation (1 novel and 1 reported hot spot mutation) in a family with strong family history. A missense mutation in heterozygous state was also detected in a female bleeder (obligate carrier) with very low factor VIII levels, probably due to extreme lyonization. This study is significant as the current strategy of genetic diagnosis in HA families is to screen for intron 22 and 1 inversions and if they are negative proceed with the indirect method of gene tracking analysis using the different markers of factor VIII gene, wherein approximately 10% are found to be non-informative and there is a need for the index case and other family members. The outcome of this study would enable us to give an accurate diagnosis in all the affected families. The phenotypic, clinical and genotypic correlation will also be presented.

1973

SEVERE BLEEDING DUE TO ACQUIRED HYPOPROTHROMBINEMIA-LUPUS ANTICOAGULANT SYNDROME. CAS REPORT AND REVIEW OF LITERATURE

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Background. The presence of lupus anticoagulant is associated with an elevated risk of venous and arterial thrombosis, and recurrent miscarriages as well. The lupus anticoagulant is never accompanied by a hemorrhagic diathesis unless it is associated with a second coagulation abnormality such as thrombocytopenia or hypothrombinemia,[1] this disease can present with bleeding as a consequence of lupus anticoagulant hypothrombinemia(LAHPS). LAHPS is a rare disease. *Case report.* A 29 year-old Algerian was admitted to our departement with cerebral hemorrhagic syndrome due to a serious coagulopathy; prothrombin time(PT) INR was 1.53 and the activated partial thromboplastin time (aPTT) ratio 2.5, diluted Russell viper venom tests (dRVVT) and Staclot LA assay confirm the presence of LA, Thrombocytopenia was detected. Evaluation of the cloting factors revealed decreased levels of factors II (30%). Associated findings in this patient included positive immunologic tests for systemic lupus erythematosus, a positive IgG anti-beta(2)GPI antibodies. Therapy with prednisone was commenced and his bleeding ceased. *Discussion.* we discuss the pathogenesis, diagnosis and management of LAC-HPS in patients with SLE. Literature review revealed 3 other patients with LAHPS who developed thrombosis resulting from the treatment of factor II deficiency may promote thromboembolism.[2] *Conclusion.* the lupus anticoagulant is commonly encountered in the laboratory, but acquired hypoprothrombinemiae is extremely rare. The condition is reviewed and its treatment discussed. Attention is drawn to the difficulty in diagnosing this situation, and therapeutic options are reviewed.

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1974

ACUTE HEMORRHAGIC OEDEMA OF INFANCY: CASE REPORT

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Acute hemorrhagic edema of infancy is a rare variety of cutaneous vasculitis presented in clinical triad of large bruise-like lesions, oedema and fever with mean age of affection 11 months. Pathophysiologically, it's a type of leukocytoclastic vasculitis probably mediated by immune complexes. IgA is observed in less than one third of skin biopsies of patients with AHEI. Spontaneous recovery is the rule. Possible complications are arthritis, nephritis, abdominal pain and gastrointestinal tract bleeding.

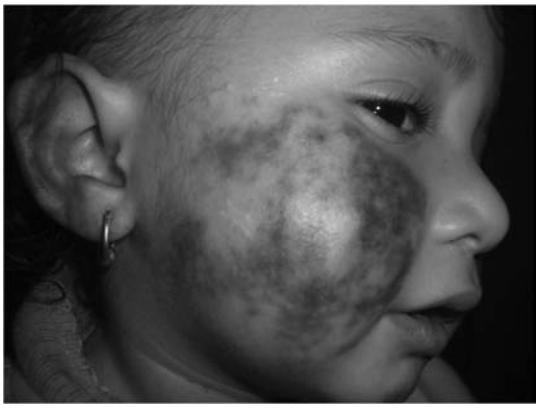

Figure. Cheeks lesions on second day of admission.

Our case is a female kid 20 months old who developed low grade fever for one day followed by purple non pruritic lesions on the cheeks and lower limbs, which became elevated, larger and tender on the following days with puffiness of the eye lids and painful mouth ulcers and bullae. There was no evidence of nephritis, arthritis nor gastrointestinal affection. She received a one week course of corticosteroids and completely resolved within two weeks with no skin scarring.

1975

CASE REPORT, CONTINUES LOW DOSES TREATMENT *VS.* ON DEMAND REGULAR DOSE TREATMENT IN CONGENITAL FVII DEFICIENCY, MORE EFFECTIVE, LESS EXPENSIVE?

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Background & Aims. Congenital factor VII (FVII) deficiency is a consequence of a genetic polymorphism that can produce a wide spectrum of disease severity. Mildly affected patients may experience increased bleeding after surgery, trauma or mucosal bleeding, while spontaneous and life-threatening bleeding occurs in patients who are severely affected. The absence of a clear-cut and consistent correlation between bleeding symptoms and FVII clotting levels is one of the main clinical features of congenital FVII deficiency. Replacement therapy is the mainstay of treatment for patients with FVII deficiency. This has traditionally been achieved using fresh frozen plasma (FFP), prothrombin complex concentrates (PCCs), or plasma-derived FVII concentrates. Recombinant activated FVII (rFVIIa) (Novo seven 1.2 mg per 2.2 mL; Novo Nordisk, Copenhagen, Denmark) is now widely used for therapy in these patients, but therapeutic schedules, optimal dosages, and administration times still have to be precisely defined. In general the recommended dose of 15-30 μg/Kg every 4-6 h, appears to be effective in maintaining homeostasis during bleeding episodes or surgery. Unlike the hemophiliacs, prophylaxis is not a common praxis in FVII-deficient patients. However, preliminary reports suggest that prophylaxis may also be effective in scenarios such as menorrhagia with iron deficiency and in patients with recurrent haemarthrosis. *Methods & Results.* We introduce a 16 y girl (BW= 40 kg) who was investigated due to menorrhagia (it was begin from first menstrual cycle in age 11 y) and found to be a case of congenital FVII deficiency (FVII:C = 3.5%).she is Moslem and because of every day bleeding she wasn't able to pray during last 4 months and also had iron deficiency anemia. First it was recommended to use rFVIIa 30 μg/kg* q12h×2day and then 30 μg/Kg/ d×5 day in each menstrual cycle. Her daily bleedings stopped and menstrual cycles became regular but between cycles she had spotting with no response to tranexamic acid. So, she was advised to use a single dose, 30 μg/kg = 1.2 mg, rFVIIa per week. With this protocol spotting stopped completely. At this time with using only two injections of rFVIIa (1.2 mg/q 12h) in the first day of menstrual cycle and weekly injections of rFVIIa (1.2 mg/w) she experiences a normal life style without any bleeding. With using the recommended dose of 15-30 μg/kg rFVIIa every 4-6 h, she must use 4-6 vials 1.2 mg of rFVIIa per day in first days of each menstrual cycle, while she only uses 5 vial 1.2 mg per month. *Conclusions.* It seems that for some patients, continues low doses treatment may be more effective and much cost benefit than recommended doses and also with decreasing the number of injections it can increase the quality of life. *According to her body weight, for prevention of waste and using the total amount of drug in a vial, it is the least dose which she can use.

1976

HEMATURIA IN A YOUNG PATIENT WITH SEVERE HEMOPHILIA AND INHIBITOR PRESENCE RECEIVING PROPHYLACTIC TREATMENT WITH RECOMBINANT FACTOR VIIA: CASE REPORT

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Hemophilia A is an X-linked congenital bleeding disorder resulting from a deficiency of factor VIII (FVIII). Therapy is based on the replacement of FVIII to hemostatically adequate levels for the prevention or treatment of bleedings. Patients with severe hemophilia are at risk for developing inhibitors to FVIII, the incidence of which is reported to be as high as 50% in patients with severe hemophilia. In such cases, agents that bypass the endogenous pathway of coagulation cascade are required. Most commonly used bypassing agents are recombinant factor VIIa (rFVIIa) and activated prothrombin complex concentrates, such as factor eight inhibitor bypassing activity (FEIBA®). Both agents can be used not only to control bleeding episodes but also as prophylaxis, in order to prevent or reduce joints bleeds and lower the risk of arthropathy in haemophiliac patients with inhibitors. The present case report concerns a 14-year old boy with severe hemophilia A and high titre inhibitor presence, who demonstrated two episodes of hematuria shortly after initiation of prophylactic treatment with rFVIIa (90μg kg-1 daily). During the first episode he presented with macroscopic hematuria, lumbar back pain and vomiting. Laboratory examination demonstrated the presence of urinary tract infection while kidney function tests and renal ultrasound were normal. The episode resolved with antibiotic treatment, hydration and decubitus, and rFVIIa prophylaxis was restarted. Three months later the patient developed a second episode of macroscopic hematuria. This time no apparent cause could be identified. The patient was managed successfully with hydration and decubitus; however, following the episode, rFVIIa treatment was discontinued and the patient was put on FEIBA prophylaxis (100U kg-1 three times weekly). He has been well and free of hematuria for over a year. rFVIIa has repeatedly and successfully been used in both congenital and acquired hemophilia for the management of bleeding episodes, including episodes hematuria. This is the first case of a hemophilia patient to be reported to present with haematuria while receiving intermittent doses of rFVIIa. Two cases previously described concerned adult patients receiving rFVIIa as a continuous infusion in order to treat bleeing from other sites. The authors suggested that mucosal bleeds, such as haematuria, are characterized by high fibrinolytic activity locally and may require higher peak levels of rFVIIa to generate sufficient thrombin in order to achieve and sustain hemostasis. One could, additionally, take into account the different pharmacokinetics of rFVIIa in the paediatric and adult population. Controlled studies are needed to determine the frequency of secondary mucosal bleedings in patients with hemophilia and inhibitors treated with rFVIIa and the optimal dose of rFVIIa, both in children and adults.

1977

RECOMBINANT FACTOR VIIA AND ACTIVATED PROTHROMBIN COMPLEX CONCENTRATE GIVEN AS SEQUENTIAL THERAPY TO A PATIENT WITH SEVERE HAEMOPHILIA A AND INHIBITOR DEVELOPMENT PRESENTING WITH A LIFE THREATENING HEMORRHAGE

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Patients with haemophilia A are at risk for developing inhibitors which render conventional factor replacement therapy ineffective. In such cases, bypassing the inhibitor is required to achieve haemostasis. Both activated prothrombin complex concentrates (aPCC) and recombinant factor VIIa (rVIIa) have demonstrated efficacy in treating bleeds in patients with inhibitor presence. However, clinical response may be variable between patients or between bleeding episodes and, in some cases, either therapeutic intervention may fail. Sequential use of multiple bypassing agents has been shown *in vitro* to provide a mechanism to increase efficacy but has not met with wide clinical use because of concern regarding the possibility of thrombosis. The present case report concerns a 16year old boy with severe haemophilia A, haemophilic arthropathy and inhibitor presence who was admitted to the paediatric surgery department for central venous catheter insertion in order to undergo immune tolerance induction. Despite prophylactic intravenous treatment with 90 mcg/kg of rVIIa given every 2 hours and 1g of tranexamic acid given every 8 hours, the patient developed a huge neck hematoma on the second post-operative day. Within a few hours the patient developed severe dyspnoea and had to be incubated and transferred to the intensive care unit. Due to refractory bleeding, a combined bypassing agent therapy was decided. The initial regimen consisted of 3 doses of rVIIa (90 mcg/kg) given every 2 hours followed by 1 dose of FEIBA (50 U/kg). FEIBA is the only aPCC presently on the market. Sequential therapy was administered with careful monitoring of the patient's clinical condition and frequent laboratory screening, evaluating the platelet count, fibrinogen and D-dimers to assess for thrombosis and DIC. The treatment regiment proved both effective and safe for the young patient, as the bleed was finally controlled while no systemic reaction or thrombogenicity was noted. Intensive therapy was weaned by reducing the number of rFVIIa and aPCC doses as the patient demonstrated clinical and laboratory improvement. Even a simple surgical procedure, as a central catheter insertion, can prove to be life-threatening for patients with hemophilia who have developed inhibitors. Sequential treatment with aPCC and rFVIIa can be used to

treat bleeds in patients failing monotherapy, however, with some concern. Large controlled studies are still needed to confirm efficacy and safety of this therapeutic approach, as well as to determine the best treatment regimens.

1978

THE CONTRIBUTION OF THE BALANCE OF HEMOSTASIS IN MONITORING THE PREGNANCY

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Introduction. Pregnancy is a physiological condition which has many biological changes. The most significant are observed in the blood count and coagulation parameters disorders of hemostasis is a major cause of morbidity and maternal mortality. *Aim.* assess the impact of these disorders, etiologies and to propose a monitoring of pregnancies and support its rapid hemostasis disorders. *Materials and methods.* This is a retrospective study over a period of 5 years (2001-2006) including all patients admitted for disorders of hemostasis in peripartum reanimation medicale, CHU BATNA ,46 patients were recruited; the inclusion criteria: the parturients presented with a disorder of hemostasis during pregnancy, any woman with a disorder of haemostasis prior to pregnancy is excludedAll patients had received blood count with a platelet fibrinogen, prothrombin time (PT kaolin partial thromboplastin time (TCK)in case of pathological results, we proceeded to assay D-dimer and the degradation products of fibrinogen (PDF). *Results.* The average patient age was 22years, with a predominance of primipares74%, 80% of these pregnancies are not followed The majority of our patients who delivered vaginally. The etiologies were dominated by: *HELLP syndrome in primipares 85%. *The post partum haemorrhage among big multiparous). 15%. Overall mortality was 20%, Linked to delay care, not followed obstetrical. In our series there was a high prevalence of eclampsia occurring in young pregnant women not followed. The existence of disorders of hemostasis during eclampsia is a prognostic factor in imposing their diagnosis by a systematic review and the immediate interruption of pregnancy. *Conclusion.* The balance of hemostasis is not indicated routinely. Each patient should examined clinical ly oriented research evidence suggestive of a disorder of hemostasis. In case of suspicion of an anomalyan assessment of hemostasis will be charged (platelet count, platelet function, quick, INR, APTT, fibrinogen). We will deepen the exploration results in pathological cases. Monitoring of pregnancies and support its rapid hemostasis disorders will reduce morbidity and maternal mortality.

1979

THE PREVALENCE OF THE MOST COMMON CAUSES FOR PRIMARY THROMBOPHILIA AMONG SAUDI PATIENTS ATTENDING THE ANTICOAGULANT CLINIC

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Objectives. The purpose of our study was to determine whether the activated protein C resistance (APC resistance) Factor V Leiden, and Prothrombin Mutation as being the most common inherited risk factor for venous thrombosis among Saudi patients attending an anticoagulant clinic. *Methods.* The study describes the results of screening all patients attending our anticoagulant clinic with a history of proven recurrent venous thromboembolism (VTE), pulmonary embolism, the first spontaneous life threatening thrombosis or at an unusual site, and patients with unexplained repeated abortion. The test done were Antithrombin (AT), Protein C (PC), Protein S and activated Protein C resistance (APC). Molecular testing to detect the mutation of factor V Leiden (FVL) and Prothrombin G20210A and MTHFR C677T mutation. A total of 3,875 patients were referred . 580 patients have been fulfilled the criteria of the study, between October 1998 to November 2008, age (14-51 years old) and 9 patients were neonates. This study was conducted at King Abdul Aziz University Hospital, Jeddah, Kingdom of Saudi Arabia. *Results.* Summarize in the Table. *Conclusion.* Factor V Leiden is the most common cause of inherited thrombosis among Saudi patients followed by Protein S. Homozygous Protein S & C deficiencies are a serious cause of extensive thrombosis with high mortality during neonatal period, an affected neonate is a marker for a group at a high genetic risk. High prevalence MTHFR mutation among repeated abortion which need a further clinical studies.

Table. Summarize the causes of inherited thrombosis.

Table: Summarize the causes of inherited thrombosis among Saudi population in Jeddah, with DVT/PE and recurrent abortion.

Tests	Total Positive	Neonate	DVT/PE	Recurrent Abortion
Factor V leiden	117	0	100	17
Protein S	68	5	58	5
Protein C	31	4	25	2
AT	0	0	0	0
Prothrombin G20210A	5	0	5	0
MTHFR C677T	210	0	30*	180
Undiagnosed	48	0	48	0
Others	101**	0	101	0
Total	580	9	367	204

*Associated with heterozygous FVL or protein S deficiency. **associated with malignancy, SCA, renal disease, infection and intensive patients

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1980

HYPERCOAGULABILITY IN BETA-THALASSEMIA AND SICKLE CELL DISEASE

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Background. Thalassemia and sickle cell disease (SCD) represent the most common forms of hereditary hemolytic anemia and result from a partial or complete lack of synthesis of one of the major β- or β-globin chains of hemoglobin A or from a single amino acid mutation (b6Glu fi Val) of the β-globin chain, respectively. Although they have different pathophysiologies, patients with these conditions manifest both biochemical and clinical evidence of hypercoagulability. While the frequency of various thrombotic complications may vary in β-thalassemia and homozygous SCD (sickle cell anemia-SCA), patients with both diseases manifest decreased levels of natural anticoagulant proteins, as well as increased levels of D-dimer (fibrinolysis). *Aim.* The aim of this study was to determine % activity of natural anticoagulant proteins, and plasma levels of fibrinogen and D-dimer in patients with β- thalassemia major ,β-thalassemia intermedia and sickle cell anemia. *Methods.* We evaluated in 59 patients (mean age 54.9±16.6 years, 33 with β- thalassemia major, 8 with β-thalassemia intermedia and 18 with sickle cell anemia) the % activity of protein C (PC-chromogenic method), free protein S (free PS-immunoassay), antithrombin III (AT III-chromogenic method) as well as plasma levels of fibrinogen (Fib-Clauss method) and D-dimer (D-D-turbidimetric immunoassay). A control group of 30 age-matched healthy subjects were studied simultaneously. Results were statistically analysed using Student's t test.

Table 1. Activity of PC, free-PS, AT III and plasma levels of Fib and D-D in patients with β- thalassemia major, β-thalassemia intermedia, sickle cell anemia and healthy subjects.
***(p<0,05).**

Group	N	PC %	Free-PS %	AT III %	Fibrinogen (mg/dl)	D-dimer (ng/ml)
β-thalassemia major	33	*66,84± 20,91	*66,87± 17,40	97,07± 24,98	278,18 ± 70,18	*327,35 ± 377
β-thalassemia intermedia	8	*65,87± 22,38	*60,87± 7,19	*91,75 ± 21,26	245,62 ± 46,40	*282,12± 281,59
sickle cell anemia.	18	95,22± 37,66	*74,33± 23,80	103,11± 12,81	342,94± 91,52	*653,50± 600,19

Results. The results are presented in Table 1. Between patients with β-thalassemia major, β-thalassemia intermedia and healthy control group there was significant difference in % activity of PC and free-PS (P<0,05). In patients with β-thalassemia intermedia activity of AT III was significantly lower (P<0,05) compared to the healthy group. In sickle cell anemia only free-PS % activity was significantly lower (P<0,05) compared to the healthy group. Plasma levels of D-D were significantly higher (P<0,05) in all patients while plasma levels of Fib didn't present significant difference compared to the healthy control group. *Summary/Conclusions.* 1. Patients with β-thalassemia major, β-thalassemia intermedia and sickle cell anemia present low activity of natural coagulation inhibitors such as protein C, free protein S and antithrombin III. 2. Patients with β-thalassemia major, β-thalassemia intermedia and sickle cell anemia present high plasma levels of D-dimer (D-D), evidence of increased fibrinolysis. 3. In β-thalassemia and homozygous SCD [sickle cell anaemia (SCA)], patients manifest decreased levels of natural anticoagulant proteins, as well as increased levels of D-dimer, evidence of hypercoagulability.

1981

CHANGES IN PLASMA LEVEL OF ENDOTHELIAL PROGENITOR CELLS IN PATIENTS WITH STABLE CORONARY ARTERY DISEASE WITH AND WITHOUT DIABETES MELLITUS TYPE II CORRELATE WITH DRUGS

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Objective. Endothelial progenitor cells (EPCs) originating from the bone marrow play a significant role in neovascularization of ischemic tissues and in re-endothelialization of injured blood vessels. The number of EPCs decreases in patients with coronary artery disease and diabetes mellitus. The aim of this study was to investigate if the administration of medication such as statins and especially rosuvastatin, and thiazolidinediones, especially pioglitazone, in patients with stable coronary artery disease with and without diabetes mellitus type II, can modify the number of endothelial progenitor cells in peripheral blood. *Method.* Eleven patients with angiographically documented coronary artery disease were recruited. Seven patients with stable coronary artery disease without diabetes mellitus and four patients with stable coronary artery disease and diabetes mellitus (defined as the need for oral antidiabetic drug treatment or insulin use). Blood for EPCs was drawn for first time on admission (baseline). In the first group of patients, was administrated rosuvastatin for a period of one month. After one month of drug's administration, blood for EPCs was drawn again. In the second group of patients, was administrated pioglitazone for one month. After one month of drug's administration blood for EPCs was drawn again. Circulating EPCs were defined by the surface markers $CD34^+$ (CD34 expressing cells) and analyzed by flow cytometry. *Results.* The endothelial progenitor cells which were enumerated by flow cytometry, were the human CD34 expressing cells. In the group of patients with stable coronary artery disease without diabetes mellitus, were observed modification of the number on the order of 0,67 ×/mL (from 2,07×/mL to 2,74 x/mL). In the group of patients with stable coronary artery disease and diabetes mellitus, were observed modification on the order of 0,89 ×/mL (from 2,1 ×/mL to 2,99 ×/mL). *Conclusion.* The results of the study demonstrate augmentation of endothelial progenitor cells after drug's administration.

1982

ROSIGLITAZONE AMELIORATES ENDOTHELIAL DYSFUNCTION AND REDUCES LDL OXIDATION POSTPRANDIALLY IN PATIENTS WITH DIABETES MELLITUS

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Purpose. Postprandial lipemia is a well known atherogenic factor especially in patients with diabetes mellitus (DM). The exact mechanism, however, remains unclear. Thiazolidinediones are known antidiabetic drugs with favorable pleotropic metabolic effects. The aim of this study was to evaluate the effect of rosiglitazone, a peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist, in postprandial endothelial function and LDL oxidation in this patient population. *Materials-Methods.* We randomized 50 patients (23 males) 61±9 years old, with adequately controlled type 2 diabetes (HbA1c < 7%) on sulfonylureas or metformin, without clinical signs of cardiovascular disease and with a negative stress test, in two groups. Patients in Group A (n=25) were started on rosiglitazone in addition to their standard regimen at a dose of 4 mg / day, while patients in Group B (n=25) received placebo instead. Lipid profile was acquired before and 4 hours after ingestion of an oral fat load (35 % weight/volume fat emulsion at a dose of 50 gr fat/m2 body surface). Endothelial function was estimated at the same time by ultrasound measurement of brachial artery flow mediated dilation (FMD). The same measurements were repeated after 12 weeks of treatment. Oxidized LDL (oxLDL) levels were assessed at the same time intervals with a commercialy available ELISA kit. *Results.* Triglyceride concentration was found consistently elevated and LDL cholesterol was consistently decreased postprandially in both groups at the baseline state and at the follow up (P<0.05). However, these differences were not significant between the two groups. On the other hand, the postprandial FMD drop was abolished only in Group A after 3 months of the rosiglitazone intake (P<0.05 for comparison between the 2 groups). A non significant postprandial increase of oxLDL levels was noted in both groups at the baseline. However, oxLDL was decreased significantly after the fat intake only in the rosiglitazone group in the follow up (P<0.05 for comparison between the 2 groups). *Conclusions.* Rosiglitazone seems to have a beneficial effect upon the endothelial function and the oxidation of LDL in the postprandial state in patients with DM. Our findings may further support the theory about the pleotropic antiatherogenic effects of thiazolidinediones.

1983

THE IMPACT OF THE G58A POLYMORPHISM ON FIBRINOGEN A-CHAIN GENE ON FACTORS V, X AND THROMBIN IN PATIENTS WITH ADVANCED ATHEROSCLEROSIS

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Background. The G58A polymorphism on fibrinogen a-chain gene has been associated with increased fibrinogen levels in healthy individuals, but its effect on patients with coronary artery disease (CAD) regarding to the effects on thrombosis/coagulation is still unknown. In the present study we examined the impact of this polymorphism on factor V (fV), factor X (fX) and thrombin time in patients with CAD. *Methods.* The study population consisted of 395 subjects, 246 of which angiographically documented for CAD. The G58A polymorphism was detected by polymerase chain reaction (PCR) and appropriate restriction enzymes. Factor X, f(V) and thrombin time were measured by standard coagulometry techniques. *Results.* The genotype distribution was GG: 37.8%, GA: 39.4% and AA: 22.8% for patients with CAD, while GG: 33.5%, GA: 44.3% and AA: 22.2% for controls. There was a significant difference in thrombin time (sec) for CAD patients *vs.* controls (19.7±4.8 *vs.* 18.9±2.1, P<0.05), while this difference did not persist for 455G carriers *vs.* 455AA homozygotes in CAD (19.3±2.1 *vs.* 19.5±2.4, P=NS) and controls (18.9±2.0 *vs.* 18.8±2.5). In addition, fV (%) was significantly higher in CAD patients than controls (121.7±28.4 *vs.* 108.01±23.7, P=0.0011), while no difference was observed for 58G carriers *vs.* 58AA homozygotes both in CAD patients (125.3±27.7 *vs.* 126.5±30.3, P=NS) and controls (106.2±22.7 *vs.* 119.2±30.8, P=NS). Finally, no significant difference was observed in fX (%) for CAD *vs.* controls (94.0±35.4 *vs.* 91.9±14.2, P=NS), as well as 58G carriers *vs.* 58AA homozygotes in CAD (94.6±22.1 *vs.* 96.1±21.5, P=NS) and controls respectively (92.1±13.5 *vs.* 98.2±6.7, P=NS). *Conclusions.* Our findings suggest that the G58A polymorphism on fibrinogen a-chain gene does not affect significantly coagulation markers such as factors V, X and thrombin time.

1984

THE CHANGES OF FIBRINOLYSIS IN DIABETES MELLITUS TYPE 2

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Background/Aims. The aim of the study was to investigate the relations among fibrinolysis, activation of coagulation, glycemic control, hypertension, BMI and medication in DM 2 patients with normo- and microalbuminuria. *Methods.* Forty-two normoalbuminuric (NAU), 42 microalbuminuric (MAU) DM type 2 patients and 42 blood donors as control group were enrolled. All patients agreed to participate in the study and signed a written informed consent. Thrombin-activatable fibrinolysis inhibitor (TAFI), plasminogen activator inhibitor 1 (PAI-1), tissue plasminogen activator (t-PA) and prothrombin fragments 1+2

(F1+2) were assessed by ELISA in all subjects. *Results.* TAFI was significantly increased in the MAU group, PAI-1 and F1+2 were significantly increased in both groups, but t-PA wasn't elevated in either group compared to controls. There were positive correlations in the NAU: TAFI and fibrinogen (r=0.65, P=0.02), PAI-1 and triglycerides (r=0.67, P=0.01), in the MAU: TAFI and F1+2 (r=0.48, P=0.02), TAFI and systolic blood pressure (r=0.53, P=0.01), PAI-1 and BMI (r=0.43, P<0.05). *Summary/Conclusions.* We found decreased fibrinolysis in DM type 2 presenting with increased PAI-1 in both NAU and MAU as well as the increased TAFI in MAU. ACE inhibitors, statins and oral antidiabetics (OADs) led to increased t-PA, while beta blockers had TAFI-lowering effect. OADs improve hypofibrinolytic state in DM type 2 by lowering both TAFI and PAI-1. We confirmed the hypercoagulable state in patients with DM type 2 by higher F1+2.

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1985

CLINICAL MANIFESTATION OF ANTIPHOSPHOLIPID ANTIBODIES

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Most of the patients with thrombosis and miscarriages have any recognizable haemostatic disorders as acquired antiphospholipid antibodies, or hereditary hypercoagulable state, or both of them. The aim of this study is to investigate incidence, localization and trigger mechanisms of thrombosis, rethrombosis, miscarriages, and management of patients with antiphospholipid antibodies. Antiphospholipid antibodies (APA) were detected in 196 patients (117 female, 97 male), with median age of 34 years at first event. Lupus anticoagulant (LA) was detected in 160 patients, anticardiolipin antibodies (ACA) in 80, beta2 glycoprotein I (b2GPI) in 28, VDRL in 12. Seventeen patients had combine LA and ACA, and 5 had LA, ACA and b2GPI. LA was negative in 11/196 (5.6%) patients. Sixty nine of 196 patients (35%) have APA associated with hereditary thrombophilias: 52 with MTHFR, 17 with APCR (FV Leiden), per 5 with prothrombin 20210 and deficience of FXII, and 14 of them (20%) have two hereditary thrombophilias. Our group consisted of highly selected patients: 115 (58%) with 245 thromboses (average 2.1), 51 women (43.6%) with 141 miscarriages (average 2.8), 53 patients (27%) with thrombocytopenia, and 26 of them without thrombosis or miscarriages, and 12 without symptoms (6%). Our patients developed first thrombosis at the median age of 39, and first miscarriage at 31 years. Patients with isolated APA (64/127) developed first thrombosis at the median age of 40 years. They had 136 thrombotic events at presentation (average 2.1) with 10.3% of arterial thrombosis (2 TIA, 6 stroke, 3 myocardial infarction, 2 infarction of spleen, 1 peripheral artery). Thirty six of 82 women had 103 miscarriages (average 2.9) with median age of 31 years at first miscarriage. Patients with APA and hereditary thrombophilias (51/69) developed first thrombosis at median age of 36 years, with 109 thrombotic events (average 2.1) and 16.5% of arterial thrombosis (3 TIA, 8 stroke, 2 myocardial infarction, 5 peripheral artery) at presentation. Fifteen of 33 women had 38 miscarriages (average 2.5) at median age of 28. Recurrent thrombosis occurred in 70/115 patients (61%) and 52.9% of them had 3 or more thrombosis when antiphospholipid syndrome (APS) was established. Most of patients developed venous thrombosis of leg (53.5%), arms (6.5%), pulmonary embolisms (18.4%), abdominal veins (2%), arterial thrombosis (13%) and others (6.6%). Thrombosis at 24 patients was associated with surgery, trauma or immobilization (11), pregnancy or delivery (5), infections (3), malignancy (2), smoke (2), and oral contraceptives (1). When diagnosis of APS was established, patients with two or more venous thromboembolisims were treated with long-term anticoagulant therapy without recurrent thrombosis. Patients with arterial thrombosis were treated with low doses of aspirin. When diagnosis of APS was established, 15 women (29.4%) with 22 miscarriages has been treated with LMWH during pregnancy, delivery and postpartum, and one with HD of IV IgG. All of them had successfully delivered 17 times. In conclusion, prophylactic long-term anticoagulant therapy is effective in patients with APS and recurrent venous thrombosis. Prophylactic anticoagulant therapy during pregnancy, delivery, and postpartum is recommended for women with APA and previously recurrence miscarriages or thrombosis.

1986

HYPERCOAGULATION SYNDROMES IN PATIENTS WITH POLYCYTHEMIA VERA (PV) AND ESSENTIAL THOMBOCYTHEMIA (ET) WITH AND WITHOUT THROMBOSIS

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Background. Increased platelet counts in ET and elevated hematocrit in PV are considered the main causes of thrombosis. However, the correlation between thrombosis and degree of thrombocytosis or hematocrit is poor. Therefore, other mechanisms should be involved in thrombus formation in both diseases. Several studies had suggested that the different heprcoagulation syndromes may contribute to thrombus formation in both diseases. *Aims.* To study the role of antithrombin III (AT), protein C (PC) and protein S (PS) deficiency, APCR, TAFI, PAI-1, tissue factor pathway inhibitor (TFPI), lupus anticoagulant (LAC), anticardiolipin (aCL) and anti β2 GPI antibodies in thrombus formation in ET and PV. *Methods.* ET (n=23) and PV (n=31) were defined according the WHO criteria. Commercial kits were used for the measurement of the factors. Lupus anticoagulant was determined by DRVVT. Normal controls (NC) (n=26) were matches for age, gender and co-morbidities. *Results.* JAK-2 mutation was found in 87% of PV and 44% of ET patients. Thrombosis was diagnosed in 14 PV patients (5 strokes, 8 MI, 1 DVT), 7 ET patients (5 strokes, 1 MI, 1 PE) and in 0 NC. The mean levels of the factors in patients with and without thrombosis in PV and ET are summarized in Table 1.

Table 1. Plasma levels of procoagulants and anticoagulants in PV and ET.

	PV (n=31) Mean ± SD		ET (n=23) Mean ± SD		Normal Controls (n=26) Mean ± SD
	Pts. with thrombosis	Pts. without thrombosis	Pts. with thrombosis	Pts. without thrombosis	
PC (%)	84.7±15.6	83.8±19.4	102.4±17.0	105.7±20.0	116±18.5
PS (%)	77.3±14.1	75.0±13.8	94.0± 7.6	85.8±17.8	105±13.0
AT III (%)	108.0±15.7	108.1±19.0	111.4±14.8	114.3±16.9	98.5±10.0
APCR	2.86±0.25	2.81±0.3	3.06±0.1	2.8±0.35	2.49±0.9
TAFI (%)	87.6± 25.6	71.6±21.0	90.3±33.4	80.0±23.8	74.3±15.1
PAI-1 (ng/ml)	14.7±7.9	12.6±6.5	26.3±18.3	12.4±9.0	11.2±8.2
TFPI (ng/ml)	12.7±5.2	9.31±2.7	12.0±5.2	11.2±5.0	6.9±2.2
LAC	1.16±0.1	1. 2±0.07	1.22±0.13	1.19±0.07	0.99±0.1
aCL (U/ml)	9.9±4.0	7. 8±3.7	12.7±11.1	7. 9±3.8	3.6±1.9
Anti β2GPI (U/ml)	16.9±13.8	6.3±5.8	4.4±1.9	4.1±1.8	3.4±1.9

The levels of PC and PS were lower in both diseases *vs.* NC but significant only in PV (95%CI 18-37, P<0.001). Patients with and without thrombosis had similar levels. AT III and APCR were significantly higher in patients *vs.* NC in both diseases but similar in cases with and without thrombosis. TAFI and PAI-1 were higher in most subgroups of patients and also in patients with thrombosis *vs.* no thrombosis. However, TAFI was significant only in PV with thrombosis *vs.* NC and PAI-1 was significant only in ET with thrombosis *vs.* ET without thrombosis and *vs.* NC (95% CI -25.6 to -2.1, P=0.02). TFPI was significantly higher in all subgroups compared to NC and also in PV with thrombosis *vs.* PV without thrombosis (95% CI -6.3 to -0.47, P<0.024). LAC and aCL were significantly higher in all subgroups *vs.* NC (95% CI -13.5 to -4.6, P<0. 001), but were not significant in patients with thrombosis compared to patients without thrombosis. Anti β2GPI was significantly higher in PV patients *vs.* NC and in PV with thrombosis *vs.* no thrombosis (95% CI -15.4 to -0.47, P=0.021). The incidence of thrombosis in patients with and without JAK-2 mutation was the same. *Conclusions.* The significantly higher levels of PAI-1 in ET and TFPI and anti β2GPI in PV, in patients with thrombosis *vs.* no thrombosis, suggest a role of those factors in thrombus formation. The low levels of PC and PS in the patients compared to NC may indicate activation of coagulation as reported previously. The high levels of AT III and APCR were unexpected. LAC and aCL were also higher in patients compared to NC. However, the non significant difference of PC, PS, AT III, APCR, LAC and aCL betweens patients with and without thrombosis suggests that the factors do not contribute to thrombosis. TAFI, which was not studied previously, was significantly higher in patients *vs.* NC but not in patients

with thrombosis *vs.* no thrombosis and therefore its role in thrombus formation is not clear. The relatively small cohort of patients in the present and previous studies merits larger studies.

1987

HIGH CONCENTRATION OF VITAMIN C REDUCES D-DIMER GENERATION DURING IN VITRO HAEMOSTATIC PROCESS

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The most frequent cause of catheter malfunction is due to thrombotic occlusion requiring intervention. Because of the risks and costs associated with the removal and replacement of these devices, salvage of occluded catheters via thrombolysis is a preferred treatment option. Usually thrombolytic agents such as Streptokinasis or t-PA are used, however these drugs have risks and high costs. There are 14 years of experience using vitamin C to restore the flow of these devices in the by the nurses of Brazilian National Cancer Institute, where are a registry of 96,6% of efficiency in 65 interventions in pediatric infirmary. The aim of this study was design an *in vitro* model to analyze the action of vitamin C on blood thrombi, because we did not find any literature about the mechanism involved. The approach was investigate if vitamin C have any fibrinolytic action quantifying d-dimer. This study was approved by the ethics comitee of Universidade Federal Fluminense and carried out using blood from 10 healthy volunteers withdrawn after sign the informed consent and 13 pools of citrated plasma. The full blood study were performed incubating total blood diluted 1:1 in vitamin C solution and the control tubes diluted with saline solution before and one hour after clotting process and incubated in water bath at 37°C. At the end of incubation time we added Aprotinin to stop the action of proteases and the plasma or serum were separated and maintained at -80 C until d-dimer measurement. The treatment of citrated plasma pools were performed the same way, adding bovine thrombin 50 U NIH/mL with CaCl2 to allow the clot formation. D-dimer of twelve pools and eight total blood were tested, because two samples presented hemolysis compromising the analysis, and the results presented lower than control, statistically significance at P<0.01in non-parametric Wilcoxon test in samples diluted before and after clotting process in full blood and citrated plasma. Average values of d-dimer for full blood treate with vitamin C were: control 190,0±225,6 μg/L (8), test 71,6±34,8 μg/L (8) * and control195,3±259,0 μg/L(10), test 89,4±48,5 (10)* before and after clotting, repectivelly. The citrated plasma presented control129,7±47,0 μg/L (13), test 82,3 ±33,1 μg/L (12)* before clotting and control 107,8±37,2 μg/L (12), test 81,3±38,2 μg/L (12)* after clotting. An additional observation is that we could not isolate the clot from eight out of ten samples to weight the clot, in full blood samples treated before clot formation resulting in mean clot weight of 82,0 ±237,7mg and controls 594,0 ±216,9 mg, presenting Wilcoxon non-parametric test with P<0,01. Surprisingly, high local concentration of vitamin C reduces d-dimer generation as a result of its action in clotting process and not by increase the fibrinolytic process. This study was supported by FAPERJ.

1988

ETIOLOGY OF HYPERCOAGULABLE STATE IN WOMEN WITH RECURRENT FETAL LOSS IN TURKEY: A PRELIMINARY REPORT

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Background. Recurrent fetal loss (RFL) is one of the most common causes of sterility. Several studies identified thrombophilia as the principal cause of recurrent fetal loss. *Aims.* The aim of our study was to determine thrombophilia causes and their frequencies in cases admitted to our department for recurrent fetal loss and the preliminary results of our study were presented here. *Methods.* The study consists of 45 women aged 28.69±4.14 years (mean±standard deviation) (21-37 years). Patients with two or more first trimester abortion or with one or more late pregnancy loss were considered for this study. Cases with genitourinary tract abnormalities or chromosomal abnormalities were excluded from the study. These women were tested for inherited and/or acquired thrombophilia with the following tests; factor V Leiden gene polymorphism, prothrombin 20210 G → A gene polymorphism, methylene tetrahydrofolate reductase 677 C → T gene polymorphism, activated protein C resistance, antithrombin deficiency, protein C deficiency, protein S deficiency, lupus anticoagulant, anticardiolipin antibodies Ig G and Ig M, high factor VIII activity, hyperhomocysteinemia, hyperfibrinogenemia and shortened activated partial thromboplastin time. The number of fetal losses in cases was ranging between 2 and 4 (median 3). There was first trimester abortion history in all cases and 12 cases mentioned late pregnancy loss. *Results.* Our data demonstrated that 44.4% of women had one or combined thrombophilic conditions. The causes and frequencies of thrombophilia detected in our cases were shown in Table. Coclusions. As a result, our preliminary data emphasizes the role of thrombophilia in women with RFL in Turkey.

Table. The causes and frequencies of thrombophilia.

The Cause of Thrombophilia	Abnormality	Ratio (%)
Factor V Leiden gene polymorphism (n=43)	Heterozygous/Homozygous	11,6% (2,3% homozygous)
Prothrombin 20210 G →A gene polymorphism (n=43)	Heterozygous/Homozygous	2,3% (heterozygous)
Methylene tetrahydrofolate reductase 677 C →T gene polymorphism (n=20)	Homozygous	20,0%
Activated protein C resistance (n=34)	<120 second	20,6% (5,9% factor V Leiden negative)
Antithrombin deficiency (n=45)	<80%	0,0%
Protein C deficiency (n=45)	<70%	2,2%
Protein S deficiency (n=45)	<60%	2,2%
Lupus anticoagulant (n=30)	Positivity	0,0%
Anticardiolipin antibodies Ig G and Ig M (n=30)	Positivity	0,0%
High factor VIII activity (n=26)	>200%	11,5%
Hyperhomocysteinemia (n=29)	>15 μmol/l	10,3%
Hyperfibrinogenemia (n=27)	>500 mg/dl	3,7%
Shortened activated partial thromboplastin time (n=30)	<26,5 second	6,7% (3,3% normal factor VIII activity)

1989

INCIDENCE AND CLINICAL PROFILE OF APC RESISTANCE IN THE ABSCENCE OF FV LEIDEN MUTATION. A TWO YEARS EXPERIENCE

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Background. APC resistance in the absence of FV Leiden is in fact very prevalent in the general population (10-15% of individuals, as determined with the aPTT-based test), and is also associated with an increased risk of venous thrombosis (deep vein thrombosis (DVT) and pulmonary embolism (PE) principally). *Aim.* To evaluate the laboratory characteristics and clinical thrombotic profile in patients tested positive for APC resistance and not carrying FV Leiden mutation. *Patients and methods.* We retrospectively reviewed the clinical files, demographic and clinical parameters, and inherited and acquired thrombophilic risk factors (protein C (PC), protein S (PS), antithrombin (AT), antiphospholipid antibodies (APA), RPCA (determined with an aPTT-based test), detection of FV Leiden and G21210A mutation) of patients tested for hypercoagulable status from January 2007 to December 2009. *Results.* 1491 patients were tested for APC resistance and 144 (0.93%) of them showed low ratio of APC resistance (<2.0). 32 patients (22.22%) (20 female / 12 male), medium age 48.5 years (range: 21-85) tested negative for FV Leiden mutation and underlying causes were investigated. Patients characteristics and laboratory results are shown in Table 1.

Table 1.

	Patients	Gender	Age	Indication to evaluate thrombophilia	Venous thrombosis	Arterial Thrombosis
FVIII↑	9	5 Female/4 Male	48 (30-85)	3 Stroke 2 DVT 2 Inherited thrombophilia 1 Optic neuropathy 1 Assisted Reproductive Technology (ART)	2	4
APA	7	5 Female/2 Male	67 (30-77)	2 PE 2 Puerperium DVT 2 Stroke 1 Stroke + DVT	5	2
Hormonal ReplacementTherapy (HRT)	4	Female	35 (32-50)	3 ART 1 Stroke	No	No
Cancer	1	Male	65			
G20210A mutation	1	Female	49	Stroke	No	1
PS↓	2	Female	35 (20-50)	1 Stroke 1 Inherited thrombophilia	No	1
FVIII↑+APA	1	Male	55	1 DVT + PE	1	No
HRT+APA	1	Female	21	1 Bilateral PE	1	No
Unknown	6	4 Female/2 Male	44 (25-67)	3 Inherited thrombophilia 1 Stroke 1 DVT 1 Miocardial infarction	1	2

Medium FVIII levels 189.5% (range: 155%-200%). 2 patients in the group of APA positive associated systemic lupus erythematous. The number of venous and arterial thrombosis was 20 in 19 patients (59.4%); 10 arterial events and 10 venous thrombosis. In our serie arterial thrombosis is slighhtly more frequent in the group of elevated levels of FVIII, and venous thrombosis more frequent in the group of APA positive patients. *Conclusions.* 1) In our serie about one-third of all cases of FV Leiden-indepedent APC resistance similar to the refered in literature. 2) Altough it is

well known that APC resistance due to other causes different from FV Leiden increases the risk of venous thrombosis, incidence of arterial and venous events in our serie is similar. 3) Overall, plasma APC resistance is a reliable indicator of clinical risk, integrating the effects of several genetic and acquired risk factors in a single measure plasma phenotype.

1990

ENDOGENOUS THROMBIN POTENTIAL (ETP) TEST AND THROMBOTIC TENDENCY MARKERS IN PATIENTS WITH LIVER CIRRHOSIS

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Background. The role played by coagulation defects in the occurrence of bleeding in cirrhosis is still unclear. This is partly due to the lack of tests that truly reflect the balance of procoagulant and anticoagulant factors *in vivo*. Conventional coagulation tests (PT/INR and aPTT) seem unable to predict the severity of bleeding problems in patients with cirrhosis of the liver possibly because they do not adequately reflect the balance between procoagulant and anticoagulant clotting factors. Recently a test has become available to routinely measure the endogenous thrombin generation potential (ETP) by Dade Behring (Marburg, Germany). *Aim.* Comparison of ETP parameters and other coagulation markers - Antithrombin III, protein C, protein S, lupus anticoagulant, plasminogen, a2-antiplasmin, APCR, homocystein- between controls and patients with liver cirrhosis. *Methods.* 56 samples, of consecutive patients with histologically confirmed liver cirrhosis, and 30 samples of controls were investigated for ETP parameters and other coagulation markers - ATIII, protein C, protein S, lupus anticoagulant, plasminogen, a2-antiplasmin, APCR, homocystein-. We used the chromogenic method on the fully automated Behring Coagulation System (BCS) for the measurement of thrombin generation parameters. *Results.* 6 patients had alcoholic cirrhosis, 22 HCV, 5 PBC, 7HBV, 1HBV and HDV and 13 cirrhosis of unknown origin. Table. *Summary/Conclusions.* The reduction of procoagulant factors in patients with cirrhosis is compensated by the reduction of anticoagulant factors thus leaving the coagulation balance with minor changes. ETP might be normal in cirrhosis. For clinical validation of these findings, a prospective clinical trial is warranted where the results of ETP must be related to the occurrence of bleeding, to a much larger number of patients.

Table.

Parameters	Patients	Controls	P Value
Antithromb III	65.81 (23-102.5)	100 (79-122)	0.0001
protein C	67.3 (17-139)	105 (78-135)	0.0001
protein S	66 (22.8-89.2)	110 (66-157)	0.0001
lupus anticoagulant	1.05 (1-2)	1.1 (1-1.2)	0.28
plasminogen	85.7 (38-149.8)	107(79-132)	0.0001
a2antiplasmin	89.1 (41.2-150)	105 (82-115)	0.001
APCR	0.83 (0.64-0.96)	0.9 (0.65-1.02)	0.0001
homocystein	10.6 (1.3-26.8)	10 (7-13)	0.4
tlag	21.5 (12.5-51.1)	19.3(12.526.37)	0.16
tmax	84.4 (40.3-126.2)	54.3(45.5-62.1)	0.007
Cmax	78.1 (44.2-146.9)	123.5(113-134)	0.0001
ETP	284.4 (24.8-472,7)	394.7 (323-451)	0.0001

1991

PEDIATRIC ANTIPHOSPHOLIPID SYNDROME : EXPERIENCE OF A SINGLE BRAZILIAN CENTER

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Background. Antiphospholipid Syndrome (APLS) and their associated clinical features have been recognized increasingly in various autoimmune and nonautoimmune diseases. A close association between APLS and recurrent arterial and/or venous thrombosis has been supported by several retrospective studies, and it seems that antiphospholipid antibodies have a direct role in the pathogenesis of the thrombophilic state of APLS. Contrasting with APLS in adults, this condition is rare in paediatric population and the relatively low prevalence and heterogeneity in children have hampered the scientific evaluation of currently available classification, diagnostic and therapeutic modalities. *Aim.* This study analyses the clinical manifestations of the paediatric patients with primary or secondary APLS treated at our service. *Materials and methods.* We reviewed the medical records of eleven patients diagnosed with primary or secondary APLS from May, 2001 to January, 2009. The patients were identified from the presence of vascular events, either venous or arterial thrombosis, anti-cardiolipin antibodies and lupus anticoagulant. *Results.* Six of eleven patients were female, with a mean age at the onset of antiphospholipid syndrome of 9.9 years. Four (36.3%) patients had an underlying autoimmune disease (Systemic Lupus Erithematosus - SLE). Venous thrombosis in the lower extremities occurred in six (54.5%), venous hepatic thrombosis in five (45.4%) and arterial thrombosis - renal, myocardial, central nervous system - in three (27.2%). The nonthrombotic clinical manifestations included hematologic manifestations in 18.1% (autoimmune hemolytic anaemia or thrombocytopenia) and skin disorders in 9.0% (skin ulcers). Inherited prothrombotic disorder - protein C deficiency - was found in one (9.0%) of 11 patients. Laboratory investigations revealed positive anticardiolipin antibodies in 90.9% of the patients and lupus anticoagulant in 63.6%. Two patients with primary APLS had serious and rare thrombotic events, namely a myocardial infarction during anticoagulation therapy and a chronic pulmonary thromboembolism, as first manifestation of the disease. All patients with thrombosis received anticoagulation therapy (low-molecular-weight heparin or vitamin K antagonists), except the patient that had myocardial infarction who received anticoagulation therapy and aspirin. *Conclusions.* APLS is a serious condition, rare in children, with high risk of recurrence and clinical manifestations of thrombosis in unusual sites. Although until now, there is not a consensus criteria for the classification and management of paediatric APLS, the diagnosis must be remembered and investigated in cases of unexplained thrombosis in childhood to the institution of early treatment and follow-up.

Table. General characteristics of patients with APLS.

Patient	Sex	Age	Events	APS	Treatment
1	F	5y3m	RVT / HVT	Primary	Anticoagulation
2	M	9y1m	HVT / IVCT DVT / MI	Primary	Anticoagulation And Antiaggregation
3	F	14y5m	AHA / SLE / DVT Ischemic stroke Skin ulcers	Secondary	Anticoagulation
4	F	14y6m	PT / SLE	Secondary	Anticoagulation
5	F	8y4m	DVT	Primary	Anticoagulation
6	F	12y2m	Thrombocytopenia RAT	Primary	Anticoagulation
7	M	14y8m	PT	Primary	Anticoagulation
8	F	12y8m	Thrombocytopenia	Primary	Antiaggregation
9	M	10y2m	SLE / DVT / IVCT	Secondary	Anticoagulation
10	M	11y9m	HVT / IVCT	Primary	Anticoagulation
11	M	13y	HVT / IVCT / DVT SLE	Secondary	Anticoagulation

RVT - renal venous thrombosis; HVT - hepatic venous thrombosis; IVCT - inferior vena cava thrombosis; DVT - deep venous thrombosis inthe lower extremities; SLE - sistemic lupus erithematosus; MI - myocardial infarction; AHA - autoimmune hemolitic anemia; PT - pulmonary thromboembolism; RAT - renal artery thrombosis

1992

CAROTID ATHEROSCLEROSIS IN TYPE 2 DIABETES MELLITUS PATIENTS: ROLE OF CHLAMYDOPHILA PNEUMONIAE INFECTION

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Background. Infectious agents, especially the intracellular Chlamydophila pneumoniae, have been supposed to be involved in the atherosclerotic process. *Aim.* We performed a cross-sectional, multicenter, outpatient protocol to study Chlamydophila pneumoniae DNA in leukocytes measured by a real-time PCR in patients with type 2 diabetes with different degrees of atherosclerosis evaluated by carotid ultrasound. *Methods.* One hundred thirty-five consecutive type 2 diabetic patients were studied. Clinical, metabolic (HbA1c, lipids) and inflammatory (high-ultrasensitive C-reactive protein, tumor necrosis factor-alpha, interleukin-6) variables were measured. Previous clinical

macrovascular disease was registered and B-mode ultrasound was performed. Real-time PCR protocol for Chlamydophila pneumoniae (Tib Molbiol, Berlin, Germany) in a LightCycler thermocycler (Roche, Basel, Switzerland) was performed in all patients, using adequate positive and negative internal controls. *Results.* Patients mean age was 62±7 years. Mean diabetes duration was 16±9 years. Mean HbA1c was 7.1±1.1%. In relation to carotid ultrasound results, 40.7% patients presented clinical atherosclerosis, 32.5% subclinical atherosclerosis and 26.6% no evidence of atherosclerosis. All groups were homogeneous in anthropometrical data. Biochemical determinations were similar in all groups except for cholesterol and non-HDL-cholesterol levels. Patients with clinical atherosclerosis had greater carotid intima-media thickness compared to the other two groups. No Chlamydophila pneumoniae DNA was detected in any of the type 2 diabetes patients regardless of the presence of clinical or subclinical atherosclerosis. *Conclusions.* The lack of detection of Chlamydophila pneumoniae DNA in leukocytes suggests that this bacterium does not have an active systemic role in the pathogenesis of atherosclerosis in middle-aged type 2 diabetic patients, and it is not a reliable marker for atherosclerosis in high risk patients.

1993

PREVALENCE AND CHARACTERISTICS OF PULMONARY EMBOLISM IN CANCER PATIENTS: 5-YEAR EXPERIENCE AT A TERTIARY INSTITUTION IN TAIWAN

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Background. Venous thromboembolism is a major problem in cancer patients and exerts a bi-directional impact. Pulmonary embolism (PE) could be fatal and contributes significantly to the morbidity and mortality of cancer patients. However, epidemiological data from Oriental societies is limited. *Aims.* We assessed the prevalence and clinical characteristics of cancer patients with pulmonary embolism at a tertiary hospital in Taiwan. *Methods.* Patients with confirmed diagnosis of PE or cancers were identified by integrated search with International Classification of Diseases codes and keyword "pulmonary embolism" from the hospitalization database in Taipei Veterans General Hospital between January 2004 and December 2008. Prevalence, clinical characteristics, and treatment outcome were recorded and analyzed using statistical analysis as appropriate. *Results.* Totally 191 patients with PE were identified and 55 (28.8%) of them had a diagnosis of active cancer. Estimated prevalence of PE in cancer patients was 2.5/1000. Among them, adenocarcinoma of lung (5.9/1000), pancreatic adenocarcinoma (10.9/1000), and ovarian cancer (9.8/1000) were the major subtypes presented with PE. Concurrent deep vein thrombosis were detected in 17 (30.9%) patients. In contrast to the patients with PE at the time of cancer diagnosis, those with PE developed during treatment were more commonly associated with advanced or refractory disease status (P=0.009 and P=0.000). 27.3% (15/55) of PE episodes in cancer patients were fatal and resulted in a 15.1-month overall survival and 7.8-month post-PE survival. *Conclusions.* Prevalence of PE in Taiwanese cancer patients is much lower than that of Western countries. Distinct features of patients developed PE at diagnosis or during treatment are worth more attention in clinical practice.

1994

LEVELS OF NATURAL COAGULATION INHIBITORS IN SICLE CELL DISEASE

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Background. Sickle cell disease (SCD) is a common hereditary hemoglobinopathy. The clinical manifestations of SCD arise from the tendency of sickle haemoglobin, known as HbS, to polymerize at reduced oxygen tensions and deform red cells into the characteristic rigid sickle cell shape. Patients exhibit increased platelet and coagulation activation, as well as decreased levels of natural anticoagulant proteins. In addition, they are characterized by thrombotic complications. The pathogenesis of hypercoagulability is likely multifactorial, with contributions from the abnormal red blood cell (RBC) phospholipid membrane asymmetry, ischemia-reperfusion injury, and chronic hemolysis with resultant nitric oxide depletion. More studies are needed to better define the contribution of hemostatic activation to the pathophysiology. *Aim.* The aim of this study was to determine activity of natural anticoagulant proteins and plasma levels of D-dimer in patients with sickle cell disease. *Methods.* We evaluated in 58 patients, (mean age 37,5±16.6 years, 43 with homozygous and 15 with heterozygous sicle cell disease) the activity of protein C (PC- chromogenic method), free protein S (free PS- immunoassay), antithrombin III (AT III- chromogenic method) and plasma levels of D-dimer (D-D-turbidimetric immunoassay).A control group of 40 age-matched healthy subjects were studied simultaneously. Results were statistically analysed using Student's t test. *Results.* The results are presented in Table 1. PC and free-PS activity was significantly lower (P<0,05) in patients with homozygous sicle cell disease compared to healthy group. In patients with heterozygous sicle cell disease only the activity of free-PS was significantly lower (P<0,05) compared to healthy group. Plasma levels of D-D were significantly higher (P<0,05) in all patients compared to healthy group while activity of AT III didn't present significant difference. *Summary/Conclusions.* 1. Patients with sicle cell disease present low levels of activity of natural coagulation inhibitors: PC, free-PS in patients with homozygous sicle cell disease and free-PS in patients with heterozygous sicle cell disease. 2. Patients with sicle cell disease (homozygous and heterozygous) present high plasma levels of D-dimer (D-D), evidence of hypercoagulability. 3. Alterations in hemostatic system with evidence of hypercoagulability are present in sicle cell disease.

Table 1. Activity of PC, free-PS, AT III and plasma levels of D-D in patients with homozygous and heterozygous sicle cell disease and healthy subjects.

Group	N	PC (%¹)	free PS (%¹)	AT-III (%¹)	D-D (ng/ml)
Homozygous Sicle cell disease	43	*76,84±20,11	*76,83±18,30	94,05 ± 21,78	*653,50± 600
Heterozygous Sicle cell disease	15	95,22± 37,66	*74,33± 23,80	103,11± 12,81	*378,95 ± 367
Control group	40	106,1±21,43	93,47±29,42	103,34±12,65	220,48±126,6

¹ mean value % activity ± SD ² mean value in ng/ml ±SD * (p<0,05)

1995

A 5 YEAR PROSPECTIVE FOLLOW-UP STUDY IN ESSENTIAL CRYOFIBRINOGENEMIA PATIENTS

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Cryofibrinogenemia could be essential or secondary to some disease such as neoplasia, infection, thrombosis, collagen vascular disease. Our group have reported in a previous study the outcome of few essential cryofibrinogenemia during a short follow-up period. Therefore, we have reported that some of these cases were in fact false essential cryofibrinogenemia, and neoplasia have been revealed several months after the diagnosis of cryofibrinogenemia. *Purpose.* We have performed a prospective multicentric 5 year follow-up study for essential cryofibrinogenemia (2005-2009). *Results.* 21 cases of de essential cryofibrinogenemia have been diagnosed. Clinical manifestations were cutaneous (76%), arthralgia (32%), multinevritis 12%, thrombosis 11%, nephrotic syndrome 11%, myalgia 9%, fever 7%. The diagnosis was confirmed by three cryofibrinogenemia dosages and morpho-pathological examination (skin, kidney). 12/21 of cases (57%) initially considered such as essential cryofibrinogenemia have been revealed during a mean follow-up of 24 months that they were in fact cryofibrinogenemia secondary to lymphoma (5 T lymphoma and 3 B lymphoma). *Discussion et conclusion.* This prospective study have confirmes the previous reported data, strongly suggesting that some of the cryofibrinogenemia initially considered such as essential were in fact secondary to lymphoma that were diagnosed after several months de follow-up. Is that only an hazardous association, or some cryofibrinogenemia considered such as essential are precosious markers of indolent lymphoma? These data suggest that a regular methodical follow-up of these type of cryofibrinogenemia should be performed in order to study their potential evolution through neoplasia. However, the small number of patients (partial-

ly related to the low prevalence of this diseases, but also to some technical differences in laboratory detection) does not allow a formal conclusion.

1996

NT-PROBNP, HS-CRP, AND HOMOCYSTEINE AS INDEXES OF INFLAMMATION ON PROTHROMBOTIC STATE IN ANTICOAGULATED PATIENTS WITH ATRIAL FIBRILLATION. RELATIONSHIP TO CHADS2 STROKE RISK STRATIFICATION

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Background. Atrial fibrillation (AF) is associated with substantial morbidity and mortality, owing to a significantly increased risk for stroke, as well as thromboembolic events. The pathogenesis of atherosclerosis involves both haemostatic and inflammatory mechanisms and there is now considerable evidence that AF is associated with an inflammatory state. Predicting which patients with atrial fibrillation will have a stroke or systemic embolic event and identifying potential risk factors for it will assist with identifying candidates who stand to gain the most from stroke prevention therapies. CHADS2 score has become the most commonly used predictive rule in clinical practice, however, the main limitation is that this risk stratification schemes were developed and are still most often applied to AF patients not yet on oral anticoagulants (OA). *Aims.* We tested the hypothesis that plasma levels of pro-B-type natriuretic peptide (NT-proBNP), a sensitive biomarker of cardiac contractile dysfunction, high sensitivity C-reactive protein (HS-CRP), homocysteine, von Willebrand factor and fibrinogen as indexes of inflammation could be related to low and high stroke risk CHADS2 stratification schema in AF patients on OA. *Methods.* We prospectively enrolled 37 AF patients recruited from subjects receiving OA from the Anticoagulation Clinic at Hospital "Nuestra Señora de los Reyes" (Canary Island, Spain) Fasting blood samples for the analysis of the biomarkers were collected from all subjects in the semi-recumbent position after 10 min rest. Blood samples were centrifuged at 3500g for 15min after clotting (15-30 min), and serum was then frozen at minus 70-80°C until the samples were analysed. Informed consent was obtained. The diagnosis of AF was identified via presentation electrocardiogram. Comparisons of clinical characteristics between low-median *vs.* high-risk AF patients was performed using the Fisher exact test for categorical data and the Wilcoxon rank sum test for continuous data. Differences between low- to high-risk groups and biomarkers were determined using the Kruskal-Wallis test. Median (with interquartile range [IQR]) levels were determined and compared. *Results.* Preliminary results of 37 AF patients, median age 75 years (range 50-91), 17 (46%) of them stratified as high-risk CHADS2 at presentation, are reported; these patients had significantly higher median NT-proBNP levels when compared with those with low-median risk (1174 *vs.* 807 pg/mL, p .0661) although AF was not independently associated with NT-proBNP after multivariable adjustment. With respect to the HS-CRP and homocysteine and CHADS2 stroke risk stratification criteria, those with high risk had the highest levels of HS-CRP (4,9 *vs.* 4,7 IU/L, p 0.04), and homocysteine 19,2 *vs.* 12,6 µmol/l, p 0.001), respectively. *Conclusion.* Among anticoagulated AF patients, HS-CRP and homocysteine were positively correlated to stroke risk factors and prognosis. NT-proBNP levels were lower in those at low to moderate-risk of stroke and not related to prognosis. The use of another approach with inflammatory biomarkers along with a CHADS2 score as risk stratification for AF patients on OA merits further study.

1997

ROLE OF ANTI-BETA2-GLYCOPROTEIN I (ABETA2GPI) ANTIBODIES IN PATIENTS WITH ANTIPHOSPHOLIPID SYNDROME (APS). CLINICAL COURSE AND LABORATORY PROFILE

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Background. Since 2006 the medium/high titer of ab2GpI antibodies has been included as a laboratory criteria for APS. In the last revision of the laboratory criteria in this setting at least one of the following parameters must be present: lupus anticoagulant (LA), IgG and/or IgM anticardiolipin antibodies (aCL) and IgG and/or IgM ab2GpI antibodies. Furthermore, the risk for APS related events is commonly correlated with the number of positive antiphospholipid antibodies and several studies confirm that triple positivity confers the highest risk of APS-related events. *Aim.* To evaluate the laboratory profile, clinical characteristics and clinical course of ab2GpI positive patients with clinical suspicion of APS or autoimmune disorder. *Patients and methods.* We retrospectively reviewed the clinical files, demographic and clinical parameters, and inherited and acquired thrombophilic risk factors of patients having clinical criteria of APS or related clinical manifestations according to the Sapporo criteria in our center for a period of two years. *Results.* 296 patients with clinical manifestations suggesting APS were evaluated for hiperocoagulable status. 13 patients (4,4%) (5 male (M)/ 8 female (F)) with a median age of 48 years (range: 26-78) were positive for IgG and/or IgM in the ab2GpI ELISA. Only 2 patients had a predominant IgM ab2GpI isotype. 3 patients with autoimmune disorder (2 SLE) had no clinical criteria for APS, 2 of them were LA and aCL negative, the remaining patient was triple positive and presented autoimmune thrombocytopenia and aortic valve dysfunction. The remaining 10 patients had clinical and laboratory criteria of APS. Risk stratification (more than one laboratory test positive (Category I) or single test positive (Category II)), clinical data according to laboratory criteria are sumarized in Table 1. The qualifying event at diagnosis was thrombosis in 7 patients (70%). Stroke was the most frequent thrombotic arterial event (3/7). 3 patients received long-term oral anticoagulant treatment, 3 aspirin alone and 1 patient both drugs. The thromboembolic recurrence in patients with venous or arterial thromboembolism was 1 of 7 cases presented as an stroke. Nevertheless this patient had refused oral anticoagulation voluntarly. No history of malignancy and/or inherited thrombophilia were observed. *Conclusions.* 1) In spite off the limited number of patients evalutated, category I patients, specially ion of LA and ab2GpI antibodies association seems to be related with an icreade risk of thrombosis as reported in the literature. 2) Stroke is the most frequent thromboembolic event. 3) Patients positive for more than one laboratory test and at high risk of thrombosis had primary APS as shown in other series.

Table 1.

	Category I (aβ2GpI+ LA)	Category I (Triple +)	Category II (aβ2GpI +)
Gender	3 M/1 F	3 F	1 M/2 F
Age	44.5 (26-76)	53 (35-76)	48 (37-53)
Thromboembolism -Venous -Arterial	 1 patient 2 patients	 2 patients	 2 patients
Sites of thrombosis	-Venous thrombosis at lower limbs - Arterial thrombosis at lower limbs and stroke - Myocardial infarction	- Stroke	-Optic neuropathy - Bone avascular necrosis
Obstetric complications	- 3 early abortions (1)	- 3 early abortions (1)	- 3 early abortions (1)
Autoimmune disorders		- Autoimmune haemolytic anemia	
Recurrence	1 patient		

1998

IS THE DOUBLE COMPOUND HETEROZYGOSITY OF HEMOGLOBIN S/B-THALASSEMIA A RISK FACTOR FOR ABDOMINAL VENOUS THROMBOSIS?

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Background. Known risk factors that predispose to abdominal vein thrombosis are divided into two main categories: congenital (protein C, and S deficiencies, ATIII deficiency, sickle cell disease) and many acquired prothrombotic states such as hematological diseases, neoplasms, abdominal infectious and inflammatory diseases, abdominal postoperative states, hepatic cirrhosis, portal hypertension, and abdominal trauma. *Aim.* To estimate the prevalence of inherited and acquired thrombophilic risk factors in patients with abdominal venous thrombosis and to compare the risk factor profiles between Budd-Chiari syn-

dromes (BCS) and splanchnic vein thrombosis (SVT) in non cirrhotic patients. *Methods.* In this retrospective study, 16 patients with abdominal venous thrombosis were evaluated. All patients with cirrhosis were not included. The patients were divided into two groups, the Budd-Chiari group (hepatic vein, inferior vena cava thrombosis) and the splanchnic venous thrombosis group (portal, splenic, superior mesenteric veins) according to venous site involved. Hereditary and acquired thrombophilic risk factors were evaluated in all patients using the Dade Behring Sysmex 1000 System for measuring protein C, S, ATIII, activated protein C resistance and coagulation factors II, V, VII, IX, X. Further, the presence of mutations for factor V-Leiden, prothrombin G20210A, and MTHFR genes was determined using the CVD Vienna Lab Strip Assay™. In 6 patients with no known risk factors determined, electrophoresis of haemoglobin using a quantitative technique was performed to evaluate the presence of HbS as a thrombosis factor. *Results.* The most common site of thrombosis determined in our patients was SVT - 9 patients, 6 had BCS, and one had mixed venous thrombosis. The acquired risk factors were significantly more common in the SVT group (SVT *vs.* BCS: 56% *vs.* 17%, P<0,05) while the hereditary risk factors had no significant differences (SVT *vs.* BCS: 11% *vs.* 33%). No risk factors were identified in 50% of patients with BCS and in 33% of patients with SVT. The patients with no risk factors identified were further tested and in 3 out of 6 haemoglobin electrophoresis was performed and it revealed double compound heterozygosity for HbS/b-thalassemia. *Conclusion.* Hereditary and acquired risk factors play an important role in the pathogenesis of abdominal venous thrombosis. Other than the known risk factors of thrombosis, sickle cell disease is a well documented factor for thrombosis at various vasculature sites. Our study rises the question of how probable could be that a double compound heterozygosity of known risk factors in the homozygous state would cause under circumstances abdominal thrombosis. After these findings further investigation has started in order to establish possible new thrombosis factors in cases where the current risk factors cannot help to establish a diagnosis especially in areas where thalassemia and sickle cell disease are prevalent.

Table 1. Sites of abdominal thrombosis and their prevalence.

	Patients w/ no risk factors	Patients w/ risk factors		Total of patients
		hereditary	acquired	
SVT	3 (33%)	1 (11%)	5 (56%)	9
BCS	3 (50%)	2 (33%)	1 (17%)	6
Mixed thrombosis	-	-	1 (100%)	1

BCS: Budd-Chiari syndromes, SVT: splachnic vein thrombosis.

1999

A NEW CASE OF DYSFIBRINOGENEMIA ASSOCIATED WITH THROMBOSIS

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Background. Fibrinogen, a 340 kDa glycoprotein, is composed of three sets of different polypeptide chains termed Aα, Bβ, and γ. It plays a crucial role in blood coagulation and it was found to be a thrombotic risk factor. Several mutations in the fibrinogen, associated with thromboses, were reported worldwide. *Aims.* The aim of our study was to characterize a case of dysfibrinogenemia in a patient with deep vein thrombosis and pulmonary embolism, with no evidence of a common prothrombotic risk factor. *Methods.* Routine coagulation testing was performed on STA-R coagulation analyzer (Diagnostica Stago). Both, fibrin polymerization and fibrinolysis were measured by turbidimetrical method at 350 nm after addition of either human thrombin or reptilase to the patient`s plasma. Kinetics of fibrinopeptide release was measured by RP-HPLC method. Gene sequencing was performed by a Sanger method. Scanning electron microscopy (SEM) was performed on VEGA Plus TS 5135 electron microscope (Tescan s.r.o.) and the viscoelastic properties of the clot were measured using ReoRox Jr. (Medirox AB). *Results.* A 55 year-old man came to the hematological attention when deep venous thrombosis in the right calf occurred coupled with a pulmonary embolism in the right lung. The patient exhibited easy bruising after subcutaneous punctures. Coagulation testing showed decreased Clauss fibrinogen level, normal total fibrinogen level and prolonged thrombin time. No common thrombotic risk factor (the patient was negative for FV Leiden mutation as well as for prothrombin G20210A mutation) was found. The patient was former smoker and had normal weight. The patient was treated with warfarin after occurrence of thrombosis for one year. Both, thrombin and reptilase induced fibrin polymerization were significantly impaired. Kinetics of fibrinopeptide release was found to be normal. tPA-activated fibrinolysis was significantly slower and the most noticeable delay occurred during the final stages of what appeared as a diphasic lysis. DNA sequencing revealed a heterozygous point mutation in exon 5 of gene FGB causing the substitution of the Bβ Arg237Ser. SEM revealed that fibrin clot was formed by less fibrils with abrupt fibril terminations; and fibrin clot had decreased stiffness than control clot. Bβ arginine 237 was found to be high conserved in mammalian fibrinogen Bβ chain. *Conclusions.* The mutation B Arg237Ser leads to impaired fibrinolysis and abnormal clot morphology with decreased clot stiffness. It is very likely the direct cause of the thrombotic complications in this patient. *Acknowledgement.* This work was supported by a grant of The Internal Grant Agency of The Ministry of Health of the Czech Republic, number NS 9636-3/2008; by a grant of The Ministry of Health of the Czech Republic, number 2373601; and by a grant of The Academy of Sciences of the Czech Republic, number KAN200670701.

2000

THE ASSOCIATION OF FXIII VAL34LEU POLYMORPHISM WITH THE MEAN AMOUNT OF PROTEIN C AND PROTEIN S IN THROMBOTIC PATIENTS REFERRING TO IRANIAN BLOOD TRANSFUSION ORGANIZATION

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Background and aims. Replacement of Val34Leu polymorphism in subunit A of coagulation factor XIII results in the replacement of Valine with Leucine in amino acid 34. As a result of this substitution, FXIII Val34Leu polymorphism acts as a factor for individual protection against thrombosis and predisposes to intracerebral hemorrhage. For the first time in Iran, the prevalence of this polymorphism in thrombotic patients was investigated and the relation between the mean amount of protein C and protein S was determined.

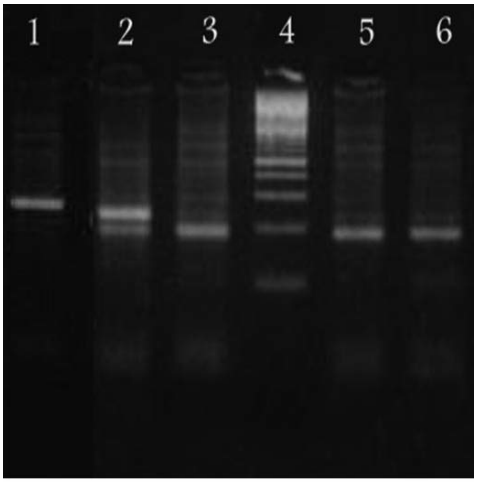

Figure.

Materials and methods. The study was performed as a retrospective case-control one. 189 referral patients with thrombotic complications were admitted to Iranian Blood Transfusion Organization (IBTO) Thrombosis and hemostasis Laboratory. The mean amount of protein C and protein S was determined in patients. Their DNA was extracted using Roche kit. Using Polymerase Chain Reaction (PCR) and RFLP methods in the presence of restriction enzyme Cfo1, genotypes of FXIII Val34Leu polymorphism were identified. The amount of protein C and protein S was determined by clotting method and STA compact instrument. Statistical analysis was performed by SPSS software version 11.5 and confidence coefficient was 95%. *Results.* The mean amount of protein C and protein S in patients who had FXIII Val34Leu polymorphism (25%) was 90.67% and 60.17%, respectively. while the mean amount of these anticoagulant proteins in patients without polymor-

phism (75%) was 113.67% and 78.98%, respectively. These results showed significant differences between the two groups (Pv=0.014). *Conclusions.* As a result significant association of this polymorphism reduced mean amount of protein C and S, it is concluded that the coexistence of FXIII Val34Leu polymorphism with these risk factors like as its coexistence with G20210A increases the risk of thrombosis. However, we recommended the larger case-control study by using of healthy individual.

2001

THROMBOPHILIA IN CONGENITAL DISORDER OF GLYCOSYLATION TYPE IA - A CASE STUDY

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Background. Congenital Disorders of Glycosylation (CDG) are severe, autossomal recessive, multisystemic metabolic diseases, characterized by abnormal biosynthesis of glycoproteins. The most common, CDG-Ia is related to deficient phosphomannomutase enzyme (PMM) activity, coded by PMM2 gene, situated in chromosomal segment 16p13. CDG-Ia has a broad spectrum of age related clinical findings with variation even within the same sibship. Most important clinical features involve the central nervous system, with development delay and hypotonia in combination with other findings that vary depending on the genotype, including coagulopathy, frequently during childhood, with low serum concentration of factors IX and XI, antithrombin, protein C and protein S. The potential for imbalance of the level of both pro and anticoagulant factors may lead to either bleeding or thrombosis. *Aims.* Present a case study of a 8 year old girl, with thrombophilia in the context of CDG-Ia. *Methods.* The patient was clinically evaluated and submitted to some diagnostic tests: coagulation studies including protein C, protein S, antithrombin and factors VIII, IX and XI, liver and thyroid function tests, measurement of serum albumin concentration, urinalysis, Carbohydrate-deficient Transferrin (CDT) measurement, Plasma Transferrin Isoelectric Focusing, Phosphomannomutase enzyme activity evaluation and molecular genetic testing. *Results.* Infant girl, without relevant family antecedents, observed by Neurology due to psychomotor delay. Followed since 9 months in Physiotherapy and Development Consultation due to hypotonia and motor acquisition delay. CDG-Ia diagnosis was made after CDT measurement (two determinations, 18,1% and 16,9%; reference <2,6% from total transferrin) and Plasma Transferrin Isoelectric Focusing. Phosphomannomutase enzyme activity in fibroblasts culture from skin biopsy was normal, being initially classified as CDG-X. However, molecular genetic testing of PMM2 showed a doble heterozygosity F157S/C241S, corresponding to a milder form of CDG-Ia. At 21 months physical examination revealed right eye convergent strabismus, axial and peripheric hypotonia, weak osteotendinous reflexes, sacrum-ilíac fat pads and absent inverted nipples. Coagulation studies were normal, aspartate aminotransferase (AST) and total T3 were increased, and urinalysis did not revealed proteinuria. At 7 years old, neurologic examination was almost normal, with discrete hypotonia. Actually, she presents a post-chirurgical right eye divergent strabismus and some difficulty to fine movements (ex: drawing), without overt clinical manifestation of thrombosis so far, although coagulation tests had revealed a decreased antithrombin (49%; reference 80-120%). Thyroid function normalized and other tests were similar to the previous results. She frequents a regular school, with acceptable results and discrete curricular adaptations. *Summary/Conclusions.* Deficiency of factor IX, factor XI, antithrombin, protein C and S can be associated to CDG-Ia and are indicators of a worse prognosis. Low levels of coagulation factors rarely cause clinical problems in daily activities, but must be acknowledged if an individual with CDG-Ia undergoes surgery, because of increased risk of bleeding and/or deep venous thrombosis. It should be possible to proceed to the prenatal diagnosis of these diseases and to appropriate genetic counseling. The CDG-Ia seems to be the first identified autossomal recessive disease in which the risk of recurrence in the family is greater than the expected for these situations.

2002

PULMONARY ARTERIAL HYPERTENSION IN β-THALASSEMIA MAJOR PATIENTS: SYMPTOM & QUALITY OF LIFE IMROVEMENT AFTER TREATMENT WITH BOSENTAN

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Background. Cardiac failure represents the leading cause of mortality in _-thalassemia major patients (TMp). Pulmonary arterial hypertension (PAH) is implicated in cases of premature death. PAH pathophysiology is complex. *Aim.* Identify TMp at risk and investigate the efficacy of bosentan (endothelin-1 receptor antagonist). *Patients & Methods.* - We retrospectively analyzed 55 TMp (mean age 34,3±7,5y, range 10-51y). After Echo-Doppler imaging, we selected 23/55 (42%) TMp with PASP>35mmHg. 28 normal & 4 TMp with overt signs of left heart disease (LVEF<55%) were excluded. - TMp with right ventricular (RV) dysfunction were further analyzed by pulmonary function test (PFTest) and 6-minute walk test (6MWD). 1 with abnormal PFTest was excluded. The others were classified according to WHO criteria: functional class-I: 10- PASP=35-45mmHg, class-II (slight limitation of physical activity): 8- PASP=46-55mmHg, class-III (marked limitation of physical activity): 4- PASP=56-70mmHg. - Treatment with bosentan was prescribed in class II & III, for 12 months, starting from 124mg and progressing to 250mg in one month. TMp were investigated at baseline and after 12 months for: PASP, 6MWD, Brain Natriuretic Peptide (BNP), ALT, AST and Quality of Life (QOL) by SF-36 Health Survey. - Statistical analysis was performed by Stata Corp., College Station, USA. - The Hospital Ethical committee approved the study. All patients provided written informed consent. *Results.* TMp were well transfused (mean Hb before transfusion=10,8 g/dL) and well chelated by combined treatment. Mean Ferritin: 251ng/dL, LIC (Ferriscan™): 1,2 mg/g/dwt, MRI-T2*L: 28,7 msec, T2*H: 34 msec. 1. Risk factors: § 8/12 males. § 11/12 splenectomized. § Mean PTL=536±222 (8/12 acetylsalicylic acid treatment). § Hereditary thrombophilia: 2/12 homozygous MTHFR, 4/12 heterozygous PAI, 5/12 double heterozygous MTHFR+PAI, 1/12 heterozygous MTHFR. § Associated complications: 7/12 Hypothyroid, 8/12 Glucose metabolism abnormalities & Diabetes, 4/12 hep C positive. 2. Follow up after 12 months of treatment: § PASP decreased: 57,2±9,2 *vs.* 49,5±7,3mmHg (P=0.002). § 6 MWT increased: 514±80 *vs.* 609±65 (P=0.002). § BNP decreased: 35,2±18 *vs.* 29±13 (P=0.007). § No increase in ALT & AST. § Improvement of QOL: SF-36, General health (8 *vs.* 7), P=0.04; Physical function (13 *vs.* 11), P=0.006; Physical role (13 *vs.* 10,5), P=0.001; Emotional role (13 *vs.* 10), P=0.006; Social function (13 *vs.* 11), P=0.006; Bodily pain (13 *vs.* 10,5) P=0.02; Vitality (10,5 *vs.* 9), P=0.004; Mental health (10 *vs.* 8,5), P=0.02. 3. Adverse events: 1 TMp discontinued treatment because of pregnancy; she delivered a healthy baby. 2 TMp reduced bosentan dose temporarily after ALT increase (>3 and ≤5 x ULN). *Conclusion.* PAH is a progressive disease leading to right heart failure and death. This is the first study that provides evidence of treatment with bosentan. Identifying and treating TMp at risk is imperative. This data indicates that bosentan is well tolerated, improves exercise ability, decreases the rate of clinical worsening and improves QOL.

2003

FACTOR FV H1299R (HR2) IN YOUNG PATIENTS WITH THROMBOEMBOLIC DISEASE

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Background and objectives. Is well known that FV Leiden involves a significant risk of venous thromboembolism (VTE). Recently, a complex haplotype of FV HR2 (M385T, H1299R, M1736V and D2194G) has been reported to be a possible risk factor for the develovement of VTE, althouh its implication is still questionable. The aim of this study is to evaluate the incidence of the Factor V gene mutation H1299R (HR2) in young patients with VTE in our area. *Desing and methods.* We designed a descriptive, retrospective study in our area (Sector ZARAGOZA III) The follow-up took place between 01/01/07 and 01/01/10. We selected 65 patients younger than 55 years old. They were screened for a classic thrombofilia study and an extended genetic study with the determination of FV HR2 to assess the risk factors associated to the different clinical manifestations of VTE. *Results.* The FV HR2 haplotype heterozygous has been identified in 9 patients with VTE. 5 men and 4

women. The thrombotic events found in our patients were: deep venous thrombosis (DVT) (5 patients), pulmonary embolism (PE) (1 patient), deep venous thrombosis and pulmonary embolism (DVT/PE) (4 patients) and recurrent superficial venous thrombosis (SVT) (1 patient). All patients with FV H1299R associated risk factors of VTE. 7 patients had acquired risk factors: immobilitation (3), oral contraceptive treatment (2), cancer (2). In 2 patients that developed spontaneous VTE were detected hereditary risk factors: FV R506Q mutation (1) and protein S deficiency (1). *Conclusions.* Factor V H1299R increased the risk of VTE in patients with others acquired or hereditary risk factors associated. Thus, further studies looking for no habitual genetic thrombophilic mutations are recommended.

2004

LABORATORY INVESTIGATION FOR THROMBOPHILIA IN PATIENTS <60 YEARS WITH ISCHEMIC STROKE

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Background. Ischemic stroke is the most common type of stroke and one of the most common causes of death worldwide. Though it is considered to be a disease of the elderly, 50% of patients are <75 years and 3% are young adults. *Aims.* The aim of this study was to detect coagulation defects in patients <60 years with ischemic stroke. *Methods.* The study included 82 patients (53 men and 29 women, mean age: 50 years). Blood samples were drawn in the first 24 hours after admission in the Neurology Department of our hospital before the initiation of anticoagulant therapy. Imaging studies of the brain (CT and/or MRI) were performed in all patients. Among these patients, 50 were diagnosed with ischemic stroke, 28 with transient ischemic attack and four with transient global amnesia. The following were considered risk factors for ischemic stroke: hypertension (HT), diabetes mellitus (DM), hyperlipidemia (HL), personal and/or family history of stroke. All patients were evaluated for protein S (PS), protein C (PC), activated protein C resistance (APCR), antithrombin III (AT), lupus anticoagulant (LA), fibrinogen and D-dimers. *Results.* In this study coagulation defects were detected in 25 cases (30%). Among these patients, 11 (13%) had abnormal APCR. In three of the above 11 patients PS deficiency was observed and in four patients factor V Leiden heterozygosity was found without any coexisting risk factors (HT, DM, HL). In nine out of the 25 patients (11%) LA was present. One of these nine patients had low levels of both PC and PS. Among those 25 patients, one patient was discovered with low PS levels as the sole abnormality. No patient had AT deficiency. Fibrinogen and D-dimers were elevated in 77% and 37% of patients respectively and are considered adverse prognostic factors. Summary/*Conclusions.* a. In the sample of patients presented in this study, 30% exhibited defects of the haemostatic mechanism. b. Laboratory investigation for thrombophilia should be performed in young and middle aged patients with ischemic stroke and it could be useful in both prognosis and treatment.

2005

COAGULATION PROFILE IN PATIENTS WITH TYPE 2 DIABETES MELLITUS AND CARDIOVASCULAR DISEASE

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Background. Vascular complications are considered the first cause of death in diabetic patients. The alterations of the hemostasis in diabetes mellitus (DM), that favor a prothrombotic state, include modifications in platelet functions, in the coagulation system and fibrinolytic sistem. Individuals with DM have an increase of the levels of factor X (Stuart-Power), fibrinogen and Von Willebrand Factor, increasing the risk of cardiovascular disease. *Aims.* The determination of the thrombotic risk in patients with type 2 diabetes mellitus compared with non-diabetic patients with cardiovascular disease, and the relation between determined parameters of coagulation. *Methods.*One hundreed eighty patients divided in 3 groups (diabetic without ischemic cardiopathy-related disorders (DM), diabetic with clinical or off-clinical (EKG, Echocord) ischemic cardiopathy-related disorders (DM+IC), non-diabetic with ischemic cardiopathy-related disorders (IC)) have been includeed in study; the patients were tested by determining three factors of coagulation profile: F X, fibrinogen and Von Willebrand Factor. *Results.* The highest level of the plasmatic fibrinogen has been observed in diabetic patients (groups DM and DM+IC) and this level is in direct relation to glycemic control; in groups DM+IC (P<0,005) and DM (P<0,05) has been observed the most powerful correlations between Von Willebrand Factor *vs.* fibrinogen. *Conclusions.* DM is by itself a major atherogenesis risk factor. The risk of developing cardiovascular events is higher in DM patients than in non-diabetic patients because hyperglycemia determines endothelial dysfunction and metabolic disorders which would explain the atherosclerosis acceleration in these patients.

2006

LOW LEVELS OF HEPARIN - RELEASABLE TISSUE FACTOR PATHWAY INHIBITOR ARE ASSOCIATED WITH INCREASED RISK OF VENOUS THROMBOEMBOLISM

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Tissue factor pathway inhibitor (TFPI) is synthesized by vascular endothelial cells and is located *in vivo* in at least four pools: approximately 75% is released into the circulation after injection of heparin, 20% circulates in blood bound to lipoproteins, 2.5% circulates free and 2.5% is present in platelets. The plasma pool (lipoprotein-bound and free) has been reported to be of less importance for protection against thrombosis than the heparin releasable pool. We therefore designed this study to investigate the relation of different pools of TFPI to venous thromboembolism (VTE). We measured plasma levels of TFBI by ELISA technique before (plasma pool) and ten minutes after intravenous injection of 7500 IU of unfractionated heparin (heparin-releasable pool) in 35 patients with history of venous thromboembolism and 20 healthy individuals. The mean plasma TFPI pool value was 71.77 + 21.15 ng/mL in VTE patients compared to controls (66.01+12.81ng/mL) (P=0.1). The mean heparin releasable pool value was 260.38 + 69.46 ng/mL in VTE patients compared to controls (385.72+74.30 ng/mL) (P=0.000). At a cut off value of 40.39 ng/mL for plasma TFPI pool in controls, we found that two out of thirty five VTE patients had values below the cut off value. At a cut of value of 237.12ng/mL for heparin-releasable pool in controls, we demonstrated that 15 out of 35 VTE patients had values below the cut off value. The likelihood ratio of low plasma TFPI pool for VTE was 4.66 (P=0.119) compared to 18.6 (P=0.021) for low heparin releasable TFPI pool. *Conclusions.* the data from the present study confirms the previous reported that the plasma TFPI pool is less important for protection against venous thrmboembolism and proves the association between low heparin - releasable TFPI pool and risk of venous thromboembolism. However, a real causative role needs further elucidation.

2007

PROTEIN C LEVEL IN BETA THALASSEMIA MAJOR PATIENTS IN EAST DELTA OF EGYPT

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Background. Thalassemic patients have increased risk for thromboembolic complications. The question arising from this is whether thromboembolic complications are the result of deficiency of protein C or not? *Aims.* We aimed to investigate the status of protein C anticoagulant pathway in thalassemia major patients and its relation to the hypercoagulable state in these patients. *Methods.* Fifty patients with β-thalassemia major; 30 non-splenectomized and 20 splenectomized and twenty healthy children as a control group, were investigated for their serum ferritin, liver enzymes, serum albumin, fibrinogen, protein C and protein S levels, thrombin antithrombin complex (TAT) and D-dimer. *Results.* Thalassemic patients had lower levels of protein C and S and higher levels of D-dimer and TAT than control group. These findings were more obvious in splenectomized patients and those with infrequent blood transfusion. *Summary/Conclusions.* Protein C plays a major role in the hypercoagulable state in thalassemic patients. These findings raise the issue whether it would be cost-beneficial to recommend prophylactic antithrombotic therapy in high risk thalassemic patients. A wider prospective study will be necessary to delineate under which circumstances this might be implicated, and at what level of protein C deficiency to start prophylactic antithrombotic therapy.

2008

PREGNANCY COMPLICATIONS AND ANTIPHOSPHOLIPID ANTIBODIES

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Background. Women with acquired and hereditary thrombophilia are at increased risk of developing venous thromboembolism and other associated gestational vascular complications like fetal loss, preeclampsia, intrauterine growth restriction, and placental abruption during pregnancy. These complications are a major cause of maternal and fetal morbidity and mortality. The primary objective of this study was to determine if elevated antiphospholipid titers or presence of lupus anticoagulant were correlated with the presence of vascular placental complications in women who admissed in obstetric consultation of tlemcen hospital, Algeria. *Methods.* The medical records of pregnant women were retrospectively collected between january 2007 and 2010. Maternal and perinatal histories including demographic data, medications, obstetric histories, and neonatal clinical manifestations and laboratory data were analyzed. We tested anticardiolipin antibodies (acl)(igg and igm isotypes)and anti-β2 gpi by enzyme linked immuno-assay (elisa), la activity (lac) using both drvv screen /confirm and staclot la. The possibility that the relationship between elevated antiphospholipid antibody titers and the outcomes of preeclampsia/eclampsie, recurrent miscarrieges, fetal death, help syndrome may have been modified by the presence of sle was evaluated in a multipl logistic regression model by creating a composite interaction term. *Results.* 51 with pregnancy complications were included. 45 of these patients had experienced frequent spontaneous abortions (88%), and five had unexplained fetal deaths (~9%). 3 had help syndrome and eclampsie, none of them had vascular thrombosis. Specific autoimmune antibodies were detected, including anticardiolipin antibody (n=8), anti-β2 glycoprotein i (n=3), and lupus anticoagulant (n=6). Women who had elevated antiphospholipid antibody titers igg had an increased adjusted odds ratio for recurrent miscarriages (P<0.01). *Conclusion.* There are several possible mechanisms by which antiphospholipid antibodies (apl) may have adverse effects on placental functions. A strong correlation between elevated igg antiphospholipid antibodies titer status and recurrent miscarriages was also found, however, whether or not there is an association between high titers of ap antibodies and preeclampsia in the absence of aps is unclear. Further investigations are needed to better understand how apl induce obstetric complications and to better clarify the functional role of heparin in the human placenta, leading to more successful therapeutic options.

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2009

DECREASED LEVEL OF HDL-CHOLESTEROL IS A RISK FACTOR FOR THROMBOSIS IN ESSENTIAL THROMBOCYTEMIA

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Background. Thrombosis represent one of the main features of essential thrombocytemia. Prior vascular events, advanced age and higher leukocyte count have been identified as risk factors for thrombosis. *Material and method.* we evaluate the role of decreased HDL- cholesterol for thrombosis at 30 patients with essential thrombocytemia associated with dyslipidemia, diabetes mellitus, obesity, hospitalized in the Clinic of Hematology from Craiova (Romania) between 2007-2009. The biological parameters evaluated were: value of haemoglobine, leukocyte count, thrombocyte count, peripheral blood smear, bone marrow smear, bleeding time, prothrombine Quick time, coagulation time, aggregation platelets tests, value of glycemia, triglyceridemia, cholesterolemia, HDL- cholesterol, LDL- cholesterol, the usual hepatic and renal tests. *Results.* Median age was 57 years, female/male ratio = 1,4, median platelet count of diagnosis was 782×10^9/L , median leukocyte count was $10,3\times10^9$/L, median value of HDL- cholesterol was 31 mg/dL. Thrombocytemia was present in all cases and leuko- cytosis in half of patients. Dyslipidemic status was present in 14 patients , obesity in 6 patients and diabetes mellitus in 5 patients. Thrombosis were present in 10 cases: Budd- Chiari syndrome in one case, coronarian thrombosis in two cases, cerebro-vascular thrombosis in two cases, deep thrombosis of the legs in three cases and deep thrombosis of the arms in two cases. All these cases associated decreased value of HDL-cholesterol < 30 mg/dL and a higher leukocyte count; six patients associated obesity and five patients diabetes mellitus. *Conclusion.* decreased HDL- cholesterol value associated with obesity, diabetes mellitus and a higher leukocyte count represent a risk factors for thrombosis in essential thrombocytemia.

2010

EXPERIENCE IN THE STUDY OF LUPUS ANTICOAGULANT (LA) WITH STC (SILICA CLOTTING TIME) AND DRVVT (DILUTE RUSELL VIPER VENOM TEST) AS DIAGNOSTIC TOOLS.

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The LA measures the ability of the antiphospholipid immunoglobulins to prolong (*in vitro*) phospholipid-dependent coagulation test. The amount and composition of phospholipids is a quantitative determinant in the assay, and it is important to reduce the number of platelets in the plasma. LA tests do not have a 100% sensitivity, therefore at least two different tests to study both coagulation ways are recommended. Due to the relative and poor specificity of LA tests, and the risk of false positives, it is extremely important to select the patients for the study. *Reason of the study.* to retrospectively analyze LA study in patients with the following conditions: thrombophilia study for artherious or venous thrombosis, women with pregnancy complications, prolonged APPT in asymptomatic patients, other (LES, inmune thrombocitopenia). The LA has been conducted following the 2009 guidelines for AL detection (J Thromb Haemost 2009;7:1737-40). Frozen PNP plasma was used as control. We define positive DRVVT a normalized ratio > 1,2, and a positive CTS a normalized ratio >1,4. *Materials and methods.* We reviewed 300 consecutive specimens of selected patients for the AL study in our Hematology department, processed between October 2009 and January 2010. *Results.* 42 patients resulted positive for LA at least in one of the tests (normalized ratio of dRVVT and TCS); the median age of the patients was 55 years old, and 75% of them were women. 13 patients suffered from thrombosis or pregnancy complications (3 of them had a known APS syndrome). We identified 2 groups (Table 1): group A included 13 patients with thrombotic events or pregnancy complications, with a median age of 42, and moderate or high titers of aCL and B2GPI in 8 cases. Group B included an older population (median age of 64 years), asymptomatic, with prolonged APPT and negative aCL and ABGPI. *Conclusions.* We identified a group of patients with clinical cryteria of APS (patients with thrombosis), presence of positive aCL, and/or positive B2GPI and positive AL test, that is coincident to high risk patients for thrombotic events. Furthermore positive LA is also observed in older asymptomatic patients without other aPL, that could match with a subpopulation of aPL without clinical significance. Waiting for more specific tests for LA, the SCT and Drvvt are an excellent combination for the study of LA, as the current guidelines state.

Table 1.

	GROUP A Trombosis events and pregancy complications (n=13)	GROUP B Asymptomatic with prolonged APTT (n=29)
Median age	42 años (20-55)	64 años (31-86)
Gender (M/F)	3/10	8/23
Known APS	3(13)	0(29)
LES	0(13)	3(29)
aCL y aB2GPI IgG/IgM Moderate/ high	8(13) Weak positive 2 (13)	0(29) Weak positive 3(29)

2011

ETIOLOGIC PROFILE AND OUTCOME OF NEONATAL THROMBOPHILIA IN EAST DELTA OF EGYPT

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Background and objectives. Thrombophilia can be defined as a predisposition to form clots inappropriately. Thrombotic events are increasingly recognized as a significant source of mortality and morbidity. The outcome of neonatal thrombophilia depends on the site of thrombosis. The presence of prothrombotic risk factors and, in particular, of the factor V Leiden mutation, is significantly associated with poor outcome after perinatal cerebral infarction; this may to predispose to hemiplegia, cerebral palsy and seizures. *Patients and methods.* This study was carried out on 30 neonates with purpura fulminans with or without evidence of venous or arterial thrombosis, detected at the neonatal intensive care unit at Pediatric Departments of Zagazig University Hospital, in east of delta of Egypt during the period from 2000 to 2008.All the patients were screened beside routine investigations for C reactive protein, as well as blood culture. They were also screened for evidence of thrombophilia thorough level of prothrombin time, thromboplastin time, D dimer, fibrin degeneration products [FDPs] as well as level of protein c, protein s, and antithrombin and factor V Leiden levels. Follow up of the patients for one year was done to evaluate psychomotor development and the recurrence of thrombophilia with radiological and laboratory investigations. Written informed consent were taken from one of parents of studied cases. *Results.* Our results revealed that hereditary thrombophilia represented 8/30 [26.7%] of neonatal thrombophilia. Factor V Leiden mutation was detected in 4/8, protein C deficiency in 2/8, Protein S in 1/8 and one case with antithrombin III. Acquired thrombophilia was 22/30 .The most common cause was septicemia 19/22. As regard of outcome, death represented with 18 cases of acquired thrombophilia, while cases suffered from cerebral palsy diplegia and seizures. Outcome of hereditary thrombophilia revealed death occurred in 2 cases while 4 cases suffered from blindness and cerebral palsy. Other two cases suffered from deformity of skin and recurrent thrombosis and seizure. *Conclusion.* Neonatal thrombophilia is considered a cause of cerebrovascular events in children. There is a link of thrombophilia with cerebral palsy.

2012

AN OPINION SURVEY ON ANTICOAGULANTS IN A LARGE NORTH LONDON NHS TRUST IN THE UK

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Background. Thromboprophylaxis and therapeutic anticoagulantion for prevention and treatment of venous thromboembolism are two issues the majority of doctors registered with the General Medical Council in the United Kingdom are expected to deal with, especially in the early stages of their professional careers. Audits of compliance with NICE guidelines for VTE prevention have become a national priority, and in the UK VTE prevention will effectively be mandated through financial incentivization. Greater than 90% of NHS Trusts are expected to demonstrate compliance with the national recommendations, but at the moment less than 50% are compliant. The National Patient Safety Agency issued an alert ("Alert 18") in March 2007 to advise healthcare organizations in the UK to take steps to manage the risks associated with prescribing, among other things, of anticoagulant therapy. In the majority of NHS trusts this task (thromboprophylaxis and initiation of therapeutic anticoagulation) falls to mainly junior doctors. Competency assessments of prescribing thromboprophylaxis and anticoagulant therapy are not necessarily carried out upon commencement of new jobs, although tools are available for this. With the introduction of new anticoagulants (direct thrombin inhibitors and anti Xa inhibitors), the tools require updating. Also, although much information is available on the new drugs it is unclear if doctors outside of specialist units are aware about their indications, uses and side effects. To get a clearer understanding of prescribing habits and knowledge about the new anticoagulants, we carried out a survey among the doctors in our NHS Trust (Barnet and Chase Farm Hospitals NHS Trust) which has a catchment population of 500,000. *Aims.* 1) To gain an insight into the anticoagulant prescribing practices of doctors. 2) To assess knowledge of new anticoagulant agents. 3) To confirm if NPSA Alert 18 recommendations with regards to prescribing are being followed. *Methods.* A simple paper questionnaire was devised with 14 questions, with answer boxes to tick. Although the questionnaire was anonymous, participants were asked to indicate their specialty and rank. The questionnaire was distributed to medical colleagues across our NHS Trust by the Haematology team comprising a specialist registrar, core medical trainee (year 2) and foundation year 1 trainee. Each returned survey was entered onto a database to allow collation of results. *Results.* So far there have been 24 returned questionnaires, with 1 rejected as it was only partially completed. We are hoping for at least 80 returns in total from all the grades and specialties in the trust to get meaningful data which would allow us to implement changes in the induction of new doctors in the trust as well as update existing doctors on new developments in thromboprophylaxis and anticoagulant prescribing in order to bring the trust in line with the recommendations from the Chief Medical Officer, National Institute of Clinical Excellence and the National Patient Safety Agency.

2013

UPSHAW-SCHULMAN SYNDROME REVEALED BY A POST PARTUM COMPLICATION; ABOUT A TUNISIAN PATIENT

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Introduction. Upshaw-Schulman syndrome is a congenital thrombotic thrombocytopenic purpura (TTP) due to mutations in the gene that encodes for ADAMTS13. It accounts for only 2-4% of all TTP cases. Many women have their first episode of TTP during pregnancy or the postpartum period. We report a case of congenital ADAMTS13 deficiency with a complex obstetric history. *Observation.* A 25-year-old woman, a previously healthy Tunisian female, issued from a non consanguineous marriage but endogamy, GI PI, was referred to our hospital at 38 weeks of gestation for gestational hypertension complicated by probable HELLP syndrome. An emergency caesarean section was performed. Afterwards, a diagnosis of TTP was made due to the combination of fever, convulsions, haemolytic anemia with presence of schizocytes (20%), peripheral thrombocytopenia and moderate renal failure. The patient was treated with repeated plasmapheresis, combined with corticosteroid therapy and oral anticoagulant with a good evolution. Further testing showed that the patient had very low (< 5% of normal) activity of ADAMTS13 and no ADAMTS13 inhibitor. The Upshaw Schulman syndrome has been retained. None of the family members (two multipareous sisters and one brother) had a history of TTP-like disorder, thrombosis, or bleeding episodes. A search for mutations in the gene for ADAMTS13 and a family inquiry are underway. Today, after a follow-up of 9 years, the patient is in sustained remission with moderate thrombocytopenia (platelet count > 100 Giga/L) and she wishes a second pregnancy! *Discussion.* TTP is difficult to diagnose, can be triggered by pregnancy, and has clinical features similar to those of other pregnancy-associated diseases such as HELLP syndrome. Therefore, the diagnosis of TTP should be considered in any patient with otherwise unexplained microangiopathic haemolytic anemia and thrombocytopenia in order to enable timely life-saving treatment. Pregnancy may precipitate TTP, presumably triggered by physiological changes. A study showed that 13% of woman diagnosed with thrombotic microangiopathy to be pregnant. According to a review of the literature, 100% of pregnant women diagnosed with congenital ADAMTS13 deficiency developed recurrent TTP in the subsequent pregnancy. What type of management plan can be constructed for today's practice for our patient, known to have congenital ADAMTS13 deficiency, who is at risk of recurrent TTP?

2014

QUALITY OF LIFE IN CHRONIC LYMPHOCYTIC LEUKEMIA AND NON-HODGKIN'S LYMPHOMA: AN ONCOLOGY CENTER STUDY OF 80 PATIENTS

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Background. Although the diagnosis of chronic lymphocytic leukemia (CLL) and non-Hodgkin's lymphoma (NHL) has a profound effect an quality of life (QOL), there are only a few studies that measure the QOL in these patients or compare it with the QOL in the general population. *Methods.* We conducted an oncology center study of patients with CLL and NHL using standardized instruments with published

population norms to evaluate fatigue and quality of life. Co-morbid health conditions were assessed using the Charlson Co-morbidity Index and we used additional questions to explore how often patients thought about their disease and special worries about it. *Results.* Between July 2009 and January 2010, 80 patients with CLL and NHL responded to the study. The physical, social/family, functional and overall QOL scores were similar to published population norms. The emotional well-being scores of CLL and NHL patients were better than the scores of patients with other types of cancer, but the majority of the patients thought about their disease daily (53%). In contrast, the emotional well-being of CLL and NHL patients were lower than the scores of general population. To determine whether emotional well-being scores improved over time, we evaluated emotional QOL among patients based on time interval since diagnosis. The emotional well-being scores improved with time and treatment. Differences in QOL were observed by stage disease, being lower among patients with advanced stage disease. Overall, the mean fatigue score was higher than population norms (2.9 *vs.* 2.2) Fatigue scores of stage A (CLL) and I A or II A (NHL) were almost identical to population norms (2.3 *vs.* 2.2), scores of advanced disease-stage C and IVB patients (3.7 *vs.* 2.2) were greater than reported for general population. Factors associated with lower quality of life included age, severity of co-morbid health conditions and current treatment. *Conclusions.* CLL and NHL have a profound effect on QOL at all disease stages. The impact of both diseases on QOL appear to differ from that of other malignancies with a less marked impact on emotional QOL, compared with literature, probably because of promising new treatments. Research identifying efficacious education and psycho-oncologic support interventions for patients with both diseases, and other malignant conditions are still needed.

2015

HOW TO MEASURE COGNITION IN HEMATOLOGICAL-ONCOLOGICAL PATIENTS?

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Background. Cognitive functions affect the quality of life of malignant disease victims. In research to date there has been a huge problem with objective measuring of cognitive abilities. Some very well-designed studies to date have not been conclusive and the authors themselves cited inadequate sensitivity of tests and equipment which were deficient in detecting minimal changes. *Aims.* The aim of research was to:1. assess to what extent malignant disease and anaemia are reflected on cognitive abilities of oncological-hemotological patients 2. define orientational norms for hematological-oncological patients, and 3. compare them with previously established norms for subjects with different educational levels and occupations using the state-of-the-art computerised equipment - a CRD instrument (Complex Reactiometer Drenovac). It is anticipated that the work will serve in designing future similar research. *Methods.* Subjects were chosen from a stratified sample of an adult patient population of both sexes who are under observation or being treated at Clinical Hospital Center, Rijeka, Croatia. A total of 400 patients divided in four commensurable groups (100 patients each) with or without malignant diseases and with or without anaemia were included in the study. The results of the psychological tests were analyzed through application of ANOVA statistical procedures. Through comparison of cognitive functions among groups, standard norms were made. *Results*. Assignment indicators in the first testing (T1) and in the repeated testing (T2) after anaemia correction, checking differences in tests among the groups as well as testing the differences between the first and second testing, demonstrated that anaemia and malignant disease are significantly connected with loss of cognitive abilities. In all assignment indicators of cognitive tests, Group 1 (with malignant disease and anemia) demonstrated the worst results in comparison with all other groups (P<0.001). After correction of anaemia, cognition improves but does not reach the result of the other groups. In Group 1 after treatment of anaemia, there was a significant restoration of cognitive functions (P=0.001) in all tests except for tests CRD21 (visual orientation) and CRD 341 (memorising). Readings for cognitive functions of all tests were compared with previously defined standards in various populations of people which allowed us to define a normative for oncological haematological patients. *Conclusions.* It was concluded that anaemia and malignant disease significantly affect cognition, where they have an additive effect. With anaemia treatment, cognitive abilities are remedied but they never reach the level of anaemia-free patients or general population. Norms for oncological-hematological patients were compared with previously established norms for subjects of different educational levels and occupations and they have been shown in tabulated form.

2016

A 15-YEAR REPORT ON NEW BIRTHS, SURVIVAL AND PRESENTING COMPLICATIONS OF PATIENTS WITH BETA-THALASSEMIA MAJOR LIVING IN NORTHERN GREECE

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Introduction. Life expectancy in thalassemia patients has improved over the years, reflecting dramatic changes in therapy. At the same time, national screening programs have limited the number of affected births worldwide. Aim of study: Report on new births, survival and complications in beta-thalassemia patients followed at the Thalassemia Unit of "Hippokration"General Hospital of Thessaloniki in Northern Greece. *Patients-Methods.* 163 patients participated in a retrospective study covering the last 15 years. Parameters evaluated where as follows: mean age, gender, genotype and beta gene mutation frequency, patient survival, iron overload (based on mean ferritin value of the past 5 years), disease related complications, reasons for death and number of new births. *Results.* 80 men (49%) and 83 women (51%) participated in the study. The mean age was 20.81 years (± 9.3 SD). The most frequently reported mutation was IVS1 -110 (44.4%). Survival was 86% at 30 years of age. The majority of patients (70.7%) had a mean serum ferritin level of <2.000 ng/mL. With regards to complications, a large proportion of patients presented with osteopenia or osteoporosis (80%), while a serious number presented with hypogonadism, abnormal glucose metabolism and heart failure. Thirteen deaths were reported during the 15 year period, with the major cause of death being heart disease (53.8%) and sepsis (30.7%). The number of new births during the time of study was 38, resulting mainly from absence of information (39.4%), medical mistakes concerning prenatal diagnosis or genetic counseling (36.8%) and informed choice due to religion issues. Most of the new births came as a result of interracial marriages. *Conclusions.* Survival of thalassemic patients continues to improve as a result of new therapeutic regimens but disease complications still prevail, affecting the patient's quality of life. The number of new births has decreased following the application of prenatal screening programs, however, better information of state and medical stuff is required in order to prevent new thalassemics being born.

2017

MUSCULO-SKELETAL FUNCTION ASSESMENT IN HEMOPHILIA A: A CROSS SECTIONAL STUDY IN EAST DELTA OF EGYPT

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Background. Morbidity in haemophilia A has been described predominantly in terms of musculoskeletal dysfunction and assessed by the clinical and radiological joint scores. These scores document changes in a particular joint, but do not reflect the impact of these changes on the individual in terms of his overall musculoskeletal function. The Functional Independence Score in Hemophilia (FISH) is a performance-based assessment tool used to measure the patients' musculoskeletal functional ability. *Aims.* We aimed to evaluate the musculoskeletal function in our hemophilia A patients and to assess the impact of degree of factor VIII deficiency and different replacement therapy regimens on it. *Methods.* Cross-sectional study on 120 children 80 hemophilic and 40 sex-, race-, and age-matched healthy males was conducted. Children over 5 years of age were selected. Musculoskeletal function was assesed using Functional Independence Score in Hemophilia (FISH), a performance-based instrument. FISH measures the patient's independence in performing seven activities under three categories: self-care (grooming and eating, bathing and dressing), transfers (chair and floor) and mobility (walking and step climbing). Each function is graded from 1 to 4 depending on the amount of assistance needed in performing the function. All relevant data regarding degree of factor VIII deficiency and type&frequency of replacement therapy were collected. *Results.* There were no differences in the FISH between controls and patients with mild hemophilia; however, the score was significantly higher in patients with mild hemophilia than patients with moderate or severe hemo-

philia. The most affected activities were squatting, walking, and step climbing. FISH scores were significantly higher in patients recieving regular factor VIII replacement than those recieving either irregular factor VIII replacement or fresh frozen plasma. *Summary/Conclusions.* Regular factor VIII replacement therapy is superior to fresh frozen plasma transfusion in reducing musculoskeletal dysfunction in hemophilia A patients. Also, patients with moderate and severe factor VIII deficiency are more vulnerable to have musculoskeletal dysfunction than those with mild deficiency.

2018

THE ADVANTAGES OF HOMOGENOUS ACUTE LEUKEMIA GROUPS OF PSYCHOLOGICAL SUPPORT IN CONTRAST WITH THE HETEROGENOUS GROUPS

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Background. The psychologic support group is considered the most efficient form of psychological support. The result of the group therapy is a diminuished fear at the new pacients towards the diagnosis, implicitely a raised confidence in the efficiency of therapeutical means and medical competence. It has been proven that the experience of knowing and interacting with another person suffering of a similar disease, who shares the same feelings is more valuable than an exposure made by the doctor or psychologist about a person with the same experience. *Aims.* We want to compare the efficiency and advantage of the two group types by analysing the feedback from the pacients and their relatives at the end of the group sessions, as well as the group mediators' observations. *Methods.* Since 4.5 years there have been meetings of homogenous and heterogenous support groups in the Oncological Institute, Hematology Department, Cluj-Napoca, Romania. The support groups are open. The heterogenity referrs to pacients with different age, gender, personality, cultural level, background, education, as well as to the pathology, with acces to pacients' groups regardless of their diagnosis, stage, evolution of the disease or therapy type. After two and a half years of experience in organising the groups, we decided to form homogen groups dedicated to acute leukemia. The support groups have two mediators, a psychologist and a hematologist with training of psycho-social counceling. The psychological approaches used within the support group are the informational- educational one (by which the therapeutical compliance and pacients' motivation raises and the false preconceived opinions are defeated and the pacients receive the pertinent sources of information), the cognitive- behaviourist approach (with the purpose of identification and improvment of coping, control of certain symptoms and the emotional support offered. The patients signed the informed consent and the study has the agreement of Ethic Committee of the Medicine and Pharmacy University Cluj-Napoca, Romania. *Results.* In a heterogenous group, the more participants (various in personality, disease i.e.) the more different the problems discussed and the experiences the participants share. The disadvantage of this group is represented by the fact that many times general problems are discussed, the specific disease problems being better handled in homogen groups, dedicated strictly to one pathology. The approach of a detail or a specific problem of a disease in a non-homogen group helps only one group participant, the others may feel left aside, isolated, not understood or may consider the fact that their problems are not important. Discussing in detail the specific apsects of a certain disease in the heterogenous group led to confusion, so that the pacients tried to apply the information discussed on their own disease. *Conclusion.* The acute leukemia patients consider that the more homogenous was the group, the morespecific the goals, and the more focused the intervention. The mediators of the support group consider that the heterogenous group is more difficult to facilitate. They observe that the acute leukemia patients were more active in homogenous group than in heterogenous one.

2019

EVALUATION OF ELDERLY PATIENTS HOSPITALIZED IN HEMATOLOGY DEPARTMENT

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Background. People aged 65 and over are accepted as elderly. People over this age limit could have various chronic diseases and some functional losses related to these disorders. The amount of people aged 65 years and older in Turkey is approximately 7% according to 2005 population census. *Aims.* The aim of our study is to investigate characteristics of elderly people hospitalized and treated in hematology department. *Methods.* This retrospective study consisted of 153 elderly patients hospitalized and treated in our clinic between 2004 and 2009. Mean age of cases was 73.13±5.45 years (mean±standard deviation) (65-88 years) and 93 of them were men. Mean duration of hospitalization was 16.69±16.89 days (mean±standard deviation) (2-86 days). *Results.* Diagnosis of patients with number of cases and hospitalization duration were shown in Table. Cases with hematological malignancy were 58.2% of all older patients and these cases were 76.2% of the cases when hospitalization duration was considered. According to both criteria (number of cases and hospitalization duration), acute myeloid leukemia was most common followed by multiple myeloma and chronic lymphocytic leukemia. One quarter of acute myeloid leukemia cases were secondary leukemia with poor prognosis. Febrile neutropenia was observed in 50.0% and antifungal treatment (except prophylaxis) was given 13.2% of cases with hematological malignancy during hospitalization. There were systemic diseases which could seriously affect standard treatment protocols in 17.0% of all elderly patients. *Conclusions.* As a result, hematological malignancies are serious and common problems observed in elderly patients with concomitant chronic diseases and related organ dysfunctions.

Table. Distribution of diagnosis in elderly patients.

Diagnosis	Distribution of Cases (%)	
	Case Number	Hospitalization Duration
Acute Myeloid Leukemia (n= 35) -secondary (n= 9)	22.9% (5.9%)	42.6% (13.1%)
Acute Lymphoid Leukemia (n= 5)	3.3%	3.3%
Chronic Myeloid Leukemia (CML) (n= 4)	2.6%	4.6%
Chronic Lymphocytic Leukemia (n= 16)	10.4%	9.0%
Hairy Cell Leukemia variant (n= 1)	0.6%	0.4%
Non-Hodgkin's Lymphoma (n= 4)	2.6%	4.3%
Multiple Myeloma (n= 22)	14.4%	11.4%
Waldenström's Macroglobulinemia (n= 2)	1.3%	0.5%
Severe Aplastic Anemia (n= 2)	1.3%	0.9%
Myelodysplastic Syndrome (n= 15)	9.8%	7.1%
Chronic Myeloproliferative Disorder (non-CML) (n= 9)	5.9%	3.5%
Myelodysplastic/Myeloproliferative Neoplasms (n= 2)	1.3%	0.7%
Nutritional Anemia (n= 16)	10.4%	3.4%
Autoimmune Hemolytic Anemia (n= 2)	1.3%	0.4%
Paroxysmal Nocturnal Hemoglobinuria (n= 1)	0.6%	0.7%
Agranulocytosis (n= 2)	1.3%	0.5%
Chronic Idiopathic Thrombocytopenic Purpura (n= 7)	4.6%	4.6%
Hemophilia A (n= 1)	0.6%	0.4%
Deep Vein Thrombosis (n= 1)	0.6%	0.2%
Adverse Effects of Drugs (Cytopenia, bleeding etc.) (n= 6)	3.9%	1.3%

2020

HYPERHOMOCYSTEINEMIA AND ESSENTIAL THROMBOCYTOSIS WITH NOURISHING DEFICIENCY

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Introduction. 154 patients between the ages of 6 and 12 years old were studied: 100 of them were boys and 54 girls, residents in urban areas and belonging to middle/upper strata of society; 85% of the cases presented moderate to severe anemia and severe thrombocytosis accompanied with microcitic elements with plasmatic homocystein highly superior to normal values. An *in situ* inspection was carried out. Every chid´s house, life conditions corresponding to the family circle: parents, brothers an sisters and everybody living in the house, their personal hygiene, their clothing and everyday´s share of food of the family group was observed. As a first step it was decided to radically change the nourishing diet as the former one consisted of carbohydrates during the seven days of the week, changing it for the ingestion of nutriments of protein origin, vegetables and diary products and the use of suitable doses of acenocoumarol. *Materials and methods.* During the first 60 days they were helped with ferrous fumarat and folic acid to raise the hemoglobin level whose value was between 8 at 9 g/dL for boys and 7 at 7,5 g/dL for girls and a minimum dose of acenocoumarol (adjusted to INR) to prevent thrombotic effects. At the next blood control, all the hematologycal values were encouragingly changing, that's why it was decided to suspend the ferrous fumarat and folic acid and acenocoumarol, this allowing the new diet to perform the corresponding supply, and to control the babies and pubescent's weight here after. *Results.* After 90 days, when the corresponding blood control was done the patients had got normal analytical values: between 11 and 11,5 g/dL for boys and 11 g/dL for girls acompanied with normocitic elements and value of count of thrombocytes between 220 and 400×10^9 and the homocystein value disminished remarkably, minor values to those stimates as refer-

ence value. *Conclusion.* All these boys and girls, by means of a diet and the period in which they received ferrous fumarat and folic acid recovered their health and vitality. Basically, it was achieved to disminish the plasmatic homocystein values to normal and manageable values to prevent future thrombotic events.

2021

IFOSFAMIDE-INDUCED SEVERE RENAL TUBULAR DAMAGE IN A CHILD WITH TWO CONSECUTIVE CANCER

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Ifosfamide is widely used in the treatment of pediatric solid tumors. Its main adverse effects are various forms of renal tubular damage, but permanent tubulopathy which requires supplementation therapy is a rare situation and became a severe problem when a second tumor which requires chemotherapy occurs. *Case.* Aim was to evaluate ifosfamide-induced nephrotoxicity on growth and renal function in children treated for two consecutive cancer. The mass which detected in the left mastoid cavity during the operation of perforated chronic otitis media was diagnosed as parameningeal rhabdomyosarcoma. The patient was treated with chemotherapy included ifosfamide (Etoposide, Vincristine, Actinomicine-D, ifosfamide, Adriamycin) and radiotherapy. Because he had hypophosphatemia, hyponatremia, glucosuria, proteinuria and polyuria, after ifosphamide therapy it was thought as tubulopathy.. Patient had neurogenic bladder due to tumor, osteomalasia and failure to thrive due to tubulopathy and treated with supplementation of potassium, sodium, phosphate by the department of pediatric nephrology and growth hormone was started by division of endocrinology for severe growth retartation (9 years old, height 104 cm (<3p), weight 17.4 kg (<3p)). After 8 years he admitted to our hospital with snoring and the mass was detected in nasopharynx and parietotemporal regions with cranial MRI. The pathological diagnosis was Burkitt's lymphoma. NHL-BFM 90 protocol was started. Antibiotic and antifungal treatment against infections was regulated according to renal function during the chemotherapy. The patient has been receiving supplementation therapy of tubulopathy for last 7 years and he has no problem about malignancy. However, he is followed with complains of developmental-growth retartation, osteomalasia, tubulopathy and neurogenic bladder. *Conclusion.* Chemotherapy included ifosfamide-induced severe renal tubular damage is a potentially progressive disease. Depending on the extent of tubular dysfunction, patients with ifosfamide-induced nephrotoxicity experienced significant growth impairment and osteomalasia. So, the investigation of tubular renal function should be part of a long-term follow-up in children after chemotherapy.

2022

THROMBOCITOPENIA AND HYPERHOMOCISTEINEMIA IN ALCOHOLIC PATIENTS

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Introduction. From December 2007 and during the following 18 months 122 patients lorry drivers working for different companies were studied. They suggested that the cause of the high number of road accidents was probably due to their habet of drinking alcohol while driving. At the start of this study it was observed: marked macrocitocis, high white blood cells count, evident thrombocytopenia, altered hepatic profile, anemia in 92% of the cases and homocysteine value highly superior to the reference value. An *in situ*s inspection was also carried out. The hygiene and housing conditions varied from one individual to another but they equaled as regards alcoholic drinks intake and diet, mainly based on carbohydrates which ranked from 5 to seven days a week. As a first step, it was decide to radically change the diet by using protein nutrients, vegetables and diary products and eliminating the dialy alcohol consumption and also fixing psychotherapy sessions. *Materials and methods.* During 90 days they were helped with folic acid to raise the hemoglobin level which was between 9 at 10 g/dL, the right dose of prednisone to avoid hepatic disorders and minimum dose of acenocoumarol to prevent thrombosis events (adjusted at INR). At the next blood control it was clear sighted that slowly and gradually all the blood values, including the hepatic profile, were encourangingly changing. That's why it was decided to follow with acenocuomarol and suspend the folic acid and prednisone, allowing the new diet to perform the corresponding supply. *Results.* After 180 days, at the corresponding hematological control, the patients achieved normal analytical values: the hemoglobin values were between 12 and 14.5 g/dL, accompanied with normocitic elements, normal white cells count, plasmatic homocysteine values within reference values and thrombocytes count placed between 220 and 400×10. *Conclusion.* After the following that allowed this study the patient's hematological levels were regained. Not only were the individuals removed from their dependence on alcoholic drinks but they also recovered a healthy life with optimal abilities and capabilities for the work they do. Besides, road accidents were also reduced to a minimun of 1%.

2023

THE OPINION OF THE PATIENTS FROM SOUTHERN TRANSYLVANIA REGARDING THE ETHICAL ASPECTS OF THE MEDICAL STAFF CONDUIT TOWARDS THE PATIENTS WITH ACUTE LEUKEMIA

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Background. Together with Romania's attending to the European Union, there was also a need to adapt our internal legislation and conduit which governs the relation medical staff - patient to the European settlements. This fact involves profound changes regarding the collaboration which was established in the last half of the century between the two parts. *Aim.* We have proposed ourselves to study in what degree this new ethical conduit applied to the patients with acute leukemia by the medical staff is liked by the patients. *Methods.* We have realized a transversal study on a group of 300 consecutive hospitalized patients from the medical departments of the Emergency County Clinical Hospital from Sibiu, who have agreed to answer at a questionnaire with 14 questions regarding the attitude which the medical staff should have in communicating the diagnosis, prognosis and treatment of the patients with acute leukemia. We have analyzed the rates of the answers, their significations and we have drawn useful conclusions for medical practice. *Results.* The medium age of the studied group was 58.26±16.82 years. The gender repartition was: 53.33% women and 46.67% men. Even though many patients with acute leukemia have reduced chances of survival, even on a short time, only 66.67% of the questioned patients considered that the diagnosis should be communicated. 98.67% of the subjects consider that the doctor is the one who should inform the patient about the cause of his illness and not the nurse or the students. 96.67% consider that the family should also be informed about the patient's diagnosis. Regarding the family members who should be informed, 54.67% of the questioned subjects consider that the wife should be informed, 25.33% consider that the sons and daughters should be informed and 20% appreciate that the parents should be informed. 61.33% believe that the patient's colleagues should be aware of the diagnosis. In the situation in which the patient would not will to know the diagnosis, 73.33% of the questioned subjects, wouldn't communicate it, and 22.67% of them would still communicate the diagnosis (4% do not know). Even though the prognosis is reserved, 80.67% of the subjects agree to be recommended to start chemotherapy or radiotherapy. The opinion regarding the naturist treatment is divided: 46.67% recommend it, 38% do not recommend it and 15.33% do not know if this method is indicated. In contrast, 60% recommend the chemotherapy too to the patients who are under naturist treatment. Regarding the associated psychotherapy, 74% of the questioned subjects, consider that this will be benefic, 10.67% do not recommend it and 15.33% are undecided. The majority (97.33%) believe that the family and colleagues should donate blood so that the patients can receive transfusions with priority. 94% from the responders consider that the sanitary education regarding the prophylaxis of exposure at factors with leukemic risk would be indicated in schools; 83.33% of them consider that a natural ecological diet, with low animal fats and normal number of calories could have a protective role against leukemia-genesis, and 87.33% believe that the daily stress could be involved in the appearance of leukemia. *Conclusions.* Although not all the questioned patients accept the communication of the diagnosis at the patients with acute leukemia, the majority agree with the conventional treatment, with the blood donation and with the application of educative measures regarding prophylaxis, diet and life style.

2024

IS SOCIOPOLITICALLY-CONFLICTIVE ISRAELI REALITY AN OBSTACLE IN THE CLINICAL TRIAL PATIENT RECRUITMENT PROCESS?

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Background. Clinical trails are vital for development of pharmaceuticals. Although patient recruitment is a fundamental step in clinical research, this process has been a relatively weak area of interest for investigators. Patient recruitment problems are the most important reason for the failure of clinical trials. Other countries have found that ethnic minorities are underrepresented in clinical trials. Israeli society is known to be steeped in sociopolitical conflict, where the minority population often rejects 'establishment' endeavors. *Aims.* We examined the possible reluctance of the research staff to propose inclusion in clinical trials to the non-Jewish (i.e. minority sector) patient, and the possible reluctance of the non-Jewish patient to participate in clinical trials. We questioned whether the lack of trust in biomedical research is more accentuated in non-Jewish patients than in Jewish patients, and whether sociopolitical reasons (i.e. anti-establishment) play a part in their decision. *Methods.* We analyzed the data registered in our data-system: the number of Jewish and non-Jewish patients to whom a clinical trial (double blind) was proposed, the percentage of acceptance and refusal in both groups and the recruitment process. *Results.* The trial enrollment rate in the Jewish population (45%) was inferior to that of the non-Jewish population (62%). *Conclusion.* Israel's minority populations were well-represented in clinical trials. Good communication skills and heightened cultural sensitivity may overcome sociopolitical obstacles in clinical trials recruitment.

2025

IS THERE A TRUE INCREASE OF NEOPLASTIC HAEMATOLOGICAL DISORDERS IN EUROPE?

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Background. Evaluating the burden of Haematological malignancies (HM) in Europe from population-based cancer registries (CR) data was a task of the HAEMACARE project. *Aims.* Evaluating prevalence of HM according to standard categories and relevant morphological subgroups based on ICDO-3 in Europe. *Methods.* Data from the EUROCARE Project database involving more than 90 CRs with period of diagnosis up to 2002 and follow up to 2003. Prevalence proportions and total number of cases at 1/1/2003 by disease duration (2,5,10, years) computed with the counting method. *Results.* Among all HM the highest prevalence is found for Non Hodgkin Lymphomas (NHL). At 5 years from diagnosis it is 63 per 100,000, ranging from 38 in Eastern to 83 in Southern European countries. Prevalence for Hodgkin's Lymphoma (HL) at 5 yrs was around 10 per 100,000 in all areas. Prevalence for Myeloid malignancies (MM) is considerably lower than NHL (20 per 100,000 overall) with higher prevalence in UK-Ireland and lower in Central Europe. Discussion and *Conclusions.* Prevalence is a cumulative indicator reflecting levels and trends of incidence, survival and population ageing. Relevant advancements have been achieved in diagnosis and treatment of HM, population-based estimates of their burden help to identify specific health needs and related costs.

This work has been partially sponsored by HAEMACARE project.

2026

THE EVALUATION OF SOCIODEMOGRAPHIC PROPERTIES OF PATIENTS WITH HEMATOLOGIC MALIGNANCY

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Background. The relationship between health and socioeconomic status is clearly described in many studies. In our country, there is no sufficient data on relationship between socioeconomic status, smoking, alcohol use, exposure to chemical substances and etiology of hematologic malignancies. Therefore, we aimed to evaluate effect of socioeconomic status on hematologic malignancies. *Material and method.* We retrospectively evaluate 135 patients who were diagnosed as a haematological malignancy between November 2004 and September 2008 in Haematology Department of Celal Bayar University School of Medicine. We then collected the demographic (gender, age, marital status, education, living space) and socioeconomic variables (job, social security status, economic indicators) and smoking, alcohol, narcotic drug usage and exposure to chemical substances of the patients. A written informed consent was obtained from all patients. SPSS 11.0 program were used for evaluating the data and Pearson Ki-square test and Student's t test were used for statistical study. *Results.* Of the patients, 59 (43%) were women and 76 (56%) were men. The ratios of living in city and rural area were 63.7% and 36.3% respectively. When compared to the population of Manisa, there was no statistical difference between them (P>0.05). With respect to education status, the ratio of patients holding a secondary school or lesser degree and patients holding an upper degree were 87.4% and 12.6% respectively, and these figures were found to be lower than the average of Turkey (secondary school and lower 83 %, upper than secondary school 17%). When hematologic malignancies were evaluated individually, educational status of patients having ALL were significantly higher compared to the other eleven diseases (P=0.01). When employment of the cases was evaluated, it was found that 89.8% of women and 52.7% of men were unemployed. The overall poverty ratio was 31.9%, with 23.2% in those living in city and 46.9% in rural places. It was observed that poverty declines in cities and also in parallel to higher education status. The poverty ratio of our cases (31.9%) being higher than the poverty ratio of Turkey (18.5%), indicated that frequency of hematologic malignancy may increase by an increase in poverty ratio. In our study, smoking was significantly higher in chronic lymphoproliferative disorders (CLPD) group than others (acute leukaemia, chronic myeloproliferative disease, myelodisplastic syndrome, lymphoma). Moreover, it was found that the ratio of smoking in CLPD patients (53.2%) was much above the average of Turkey (31.9%). This finding connotes the idea that smoking may be related to CLPD. When exposure to chemical substances was evaluated in our patients, the ratio of patients exposed to chemical substances was significantly higher in CLPD group compared to the other groups (P=0.019). *Discussion and conclusion.* Our findings suggest that poverty is strongly related to haematological malignancy and smoking and exposure to chemical substances have connection with CLPD. More prospective studies are needed in this field to evaluate the relationship between socioeconomic status and demography.

2027

THE EUROPEAN WORKING TIME DIRECTIVE -A BENEFIT OR BURDEN TO HAEMATOLOGY NURSING STAFF?

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Background. The European Working Time Directive was enacted into UK law in 1998 - seeking to protect the health and welfare of workers it gave some employers time to absorb the changes. In the N.H.S. for example Doctors hours compleid by August 2009. Nurses on our Haematology ward in University Hospital of Wales, Cardiff carried on working time practice which could be seen as at odds with the directive's recommendations - change had to evolve. How we breached regulation:- In working 2×7.5 hour day shifts (early and late) and 1×10 hour night shift staff often had only 9.75 hours break between shifts. The weekly break of 24 hour duration within 7 day period could be reduced to 2×12 hour shorter breaks. Rest breaks of at least 20 minutes (away from 'work station') became controversial. Was it paid or unpaid?

Employers tried to enforce 'unpaid' breaks which lengthened the working day, and were resisted by staff. How we modified:- Our nursing workforce called for 12.5 hour shifts. This was piloted and evaluated over 3 month period. *Aims.* To demonstrate by examination of the pilot period and anecdotally beyond that the positive / negative impact of the new nursing shift system. *Methods.* We used questionaire to staff which had some 'structured' questions and room for comments and incidental observations - from this we hoped to gain subjective data. For objective data we examined recorded sick leave prior to the period of change and compared it with the pilot period. *Results.* Staff identified less stress and paperwork to meed middle of day handover deadlines - more time for patient care. Doctors felt less of day devoted to chasing up afternoon staff, noticed that ward felt more calm and subdued. Relatives remarked that staff levels seemed improved and they appreciated speaking to nurses who were actually on doctors' a.m. rounds. Managerial finding - the same number of staff on the new rota covered more shifts at no extra cost. Rota'd to 36 hours per week not 37.5 means that every four weeks there is an extra 6 hourly shift to rota. This can be used for patient care but is flexible and often used for study days for ongoing education and updating. Also 6 hours allocated to formal study day is sufficient (the old 7.5 hours wasted more clinical time than necessary). *Summary.* In summary the law eminating from Europe in 1998 - though it has taken time to filter through has helped catalize a new shift pattern for our haematology ward nurses which has been evaluated very positively. Since the pilot, numbers on sick leave have remained lower than with the previous shift pattern which also supports the intention of the European Working Time Directive of 1998 as very much a benefit.

2028

OPTIMISATION OF HAEMATOPOIETIC PROGENITOR CELL COLLECTION BY THE INTRODUCTION OF A STANDARD REFERRAL FORM AND FORMALISATION OF THE START OF CHEMOTHERAPY AND G-CSF MOBILISATION

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The University Hospital in Cardiff performs haematopoietic progenitor cell collections for autologous and sibling donors referred from all over Wales. For many of the patients attending for haematopoietic progenitor cell apheresis (HPC-A), the collection centre had either received no referral documentation or the information on the referral was incomplete. The information that was absent ranged from priming regimen and the quality of venous access to medication likely to be of significance to the procedure, for example anticoagulation therapy. This made the logistics of efficiently utilising the two Cobe Spectra Apheresis Machines available difficult, as on many occasions collections were delayed, cancelled or not scheduled for the correct day. Even allowing for the variable response to priming therapy seen across the spectrum of patients attending, it was felt that a more formal approach to priming might improve efficiency. It was also apparent that priming regimes and G-CSF doses were dependent on the individual prescriber or local hospital policy and there was some disparity between hospitals. When G-CSF alone was used for priming, there appeared to be more priming failures or patients requiring 2-3 collections before a satisfactory harvest was obtained. However, it was unknown whether this was related to patient factors or to the logistics of ensuring collection on the optimal day. An audit was therefore undertaken, the main objectives of which were to; - Standardise and improve the referral process between the district general hospitals and the collection centre. - Standardise priming regimes and start day of G-CSF. It was envisaged that the audit would improve efficiency and contribute to JACIE compliance. Collaboration with the referring hospitals resulted in the production of a standard referral form which included clinical information and history of treatment relevant to HPC-A collection. The audit period was 18 months, of which one year was retrospective, and included all patients who underwent HPC-A collection or for whom HPC-A collection was attempted during this time. The audit period coincided with a trial of the mobilisation therapy Plerixafor on compassionate grounds. The use of Plerixafor was noted in the audit information. The preliminary findings have shown that; - The referral form was being used successfully so that the machine time could be allocated more effectively. - The regimes and G-CSF start date were standardised. - The use of Plerixafor appears to have facilitated the collection process. Ongoing analysis will determine whether patient's disease, treatment, previous transplant or chemotherapy affected the collection process in any way.

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